The role of leptin receptors in the endocrine pancreas and nucleus tractus solitarius

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Declaration of Authenticity and copyright

I declare that this work is my own unless appropriately referenced. No part of this thesis has been submitted for any other degree. Some of the results here have been presented at conferences or published in scientific journals.

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Abstract

The highly controlled regulation of pancreatic hormone secretion is vital to keep the body's glucose concentration at a constant level. Defects in the regulation of glucose levels are involved in several metabolic diseases, including type 2 diabetes and obesity.

Leptin is a satiety hormone with important roles in the maintenance of body weight and glucose homeostasis. Mice that lack leptin (*ob/ob*) or the leptin receptor (*db/db*) are massively obese and have diabetes symptoms. Leptin has been demonstrated to have an effect on glucose homeostasis that is suggested to be secondary to the obesity these animals are suffering from. Currently, it is unclear how leptin regulates glucose homeostasis.

Leptin mediates its effects by interaction with its leptin receptor (LepRb), which is highly expressed in the hypothalamus, and at lower levels in the periphery. Leptin's effect on glucose homeostasis has been proposed to be mediated via its receptor expressed on pancreatic cells affecting insulin secretion. Previous animal studies have deleted the leptin receptor in pancreatic β- and α-cells using either "leaky" or inefficient *Cre*-drivers resulting in conflicting results on glucose homeostasis. In this study, we use a β-cell selective Ins1*Cre* promoter in mice to investigate the role of leptin receptor expressed on pancreatic cells effect on glucose homeostasis. Deletion of LepRb was found to have minor effects on glucose tolerance in female animals an effect that was only detected in 8 weeks old animals. No effect was observed in male animals or in females above the age of 8 weeks.

It is well established that the LepRb in hypothalamus plays an important role in regulation of energy balance. However, the LepRb is expressed in several areas outside hypothalamus, such as the nucleus of the solitary tract (NTS). GLP-1-expressing neurons in this area express the LepRb and it is therefore possible that these neurons mediate an effect on energy homeostasis or glucose homeostasis. We have therefore deleted LepRb in GLP-1 expressing neurons with a proglucagon specific promoter iGlu*Cre*. In this study, we found no effect on body weight or glucose homeostasis in animals deleted for LepRb in GLP-1 expressing neurons.

Hypothalamus is the brain region that plays a key role in the regulation of feeding and energy homeostasis. This area contains anorexigenic and orexigenic neurons and intermingled with these neurons are sub-population of neurons named RIP2*Cre* neurons expressing insulin. Due to the neurons location in the feeding area of the brain they are most likely having a role in energy homeostasis. Previous studies have suggested that tumour suppressor LKB1 plays a role on body weight and food intake in these neurons. Therefore, we deleted LKB1 selectively in the RIP2*Cre* neurons but failed to see a difference in body weight.

Abbreviations

AAV: Adeno associated virus
ADP: Adenosine diphosphate
AGRP: Agouti-related protein
AICAR: 5-Aminoimidazole-4-carboxamide ribonucleotide
AKT: Protein kinase B
AMPK: Adenosine monophosphate-activated kinase
ANT: Adenine nucleotide translocator
A-P: Antero-posteriority
AP: Area postrema
ARC: Arcuate nucleus
ATP: Adenosine triphosphate
BBB: Blood brain barrier
BCGS: Brain centred gluco-regulatory system
BDNF: Brain derived neurotrophic factor
cAMP: Cyclic adenosine monophosphate
CART: Cocaine- and amphetamine-regulated transcript
CCK: Cholecystokinin
CRF: Corticotropin-releasing factor
CNS: Central nervous system
CREB: Involving cAMP response element-binding protein
CVO: Circumventricular organs
DAPI: 4,6-diamidino-2-phenylindole
db/db: Leptin receptor deficient
DMEM: Dulbecco's modified eagle media
DMH: Dorsomedial hypothalamus
DMSO: Dimethylsulfoxide
DPP-IV: Dipeptidyl-peptidase-IV
eGFP: Enhanced green fluorescent protein
ELISA: Enzyme-linked immunosorbent assay
EPAC2: Exchange Protein Activated by cAMP-2
ER: Endoplasmic reticulum
F/F: Heterozygot for flox'd alleles
F: Flox'd
FACS: Fluorescence-activated cell sorting
FBS: Fetal bovine serum

Flox'd: loxP-flanked

G6-P: Glucose-6-phosphate

GABA: γ-aminobutyric acid

GC: Genome copies

GDH: Glutamate dehydrogenase

GFP: Green fluorescent protein

GHS: The growth hormone secretaogue

GIP: Glucose-dependent insulinotropic polypeptide/gastric inhibitory polypeptide

GK: Glucokinase

GLP-1: Glucagon like peptide 1

GLP-1R: Glucagon like peptide 1 receptor

GLP-2: Glucagon like peptide 2

GLUT: Glucose transporter

GLUT2: Glucose transporter 2

GRPP: Glicentin related pancreatic polypeptide

GSIS: Glucose-stimulated insulin secretion

GTT: Glucose tolerance test

HG: High glucose

HGP: Hepatic glucose production

HTRF: Homogeneous Time Resolved Fluorescence

IAPP: Amylin or amyloid polypeptide

IBMX: 3-isobutyl-1-methylxanthine

iCre: improved version of *Cre*

ICV: Intracerebroventricular

IHC:Immunohistochemistry

Ins1: Insulin 1

Ins2: Insulin 2

Ip: Intraperitoneal

IP1: Intervening protein 1

IP2: Intervening protein 2

IPGTT: Intraperitoneal glucose tolerance test

IR: Insulin receptor

IRS: Insulin receptor substrate

ITR: Inverted terminal repeat

ITT: Insulin tolerance test

JAK: Janus kinase

K_{ATP}: ATP-sensitive K^+ channel

KO: Knock-out

KRB: Krebs-Ringer bicarbonate buffer

LepRb: Leptin receptor

LG: Low glucose

LH: Lateral hypothalamus

LKB1: Liver kinase B1

m3AchR: Muscarinic acetylcholine receptor

MAPK: Mitogen activated protein kinase

MCH: Melanin-concentrating hormone

Mchr: Melanin-concentrating hormone receptor

ME: Median of eminence

mIP2: Mouse insulin promoter 2

mTOR: Mammalian target of rapamycin

NHS: National Health Service

NPY: Neuropeptide Y

NTS: Nucleus of the solitary tract

O/N: Over night

ob/ob: Leptin deficient

OB: Olfactory bulb

OGTT: Oral glucose tolerance test

OXM: Oxyntomodulin

PB: Phosphate buffer

PBS: Phosphate buffered saline

PC: Prohormone convertase

PCR: Polymerase chain reaction

Pdx1: Pancreas duodenal homeobox 1

PFA: Paraformaldehyde

Phox2b: Paired-like homeobox 2b

PI3K: Phosphoinositide 3-kinase

PKA: Protein kinase A

PKC: Protein kinase C

POMC: Pro-opiomelanocortin

PP: Pancreatic polypeptide

PPARα: Peroxisome proliferator-activated receptor alpha

PPG: Preproglucagon

ProG: Proglucagon

pSTAT3: Stat3 phosphorylation

PTEN: Phosphatase and tensin homolog

PVN: Paraventricular nucleus

PYY: Peptide tyrosine tyrosine

qRT-PCR: Quantitative Real-Time PCR

RNASeq: Massive parallel RNA sequencing

RIP2: Rat insulin promoter 2

ROS: Reactive oxygen species

RPM: Rotation per minute

RT: Real time

RT-PCR: Reverse transcription PCR

SEM: Standard error of the mean

SF1: Steroidogenic factor 1

SGLT2: Sodium glucose co-transporter 2

SNAP: Synaptosomal-associated protein

SP: Signal peptide

SH2B1: Src homology 2 B adapter protein 1

STAT: Signal transducers and activators

STZ: Streptozotocin

STZ-D: streptozotocin –induced diabetic

SUR: Sulfonylurea receptor

T1D: Type 1 diabetes mellitus

T2D: Type 2 diabetes mellitus

TBS: Tris-buffered saline

tdRFP: Tandem-dimer red fluorescent protein

TRH: Thyrotropin-releasing hormone

VGLUT2: Vesicular glutamate transporter 2

VMH: Ventromedial hypothalamus

WT: Wild type

YFP: Yellow fluorescent protein

α-MSH: α-Melanocyte-stimulating hormone

Chapter 1 Introduction

The **brain** contains several neuronal circuits that are involved in detecting peripheral signals containing information of satiety and energy stores and integrating the inputs to modulate food intake, energy expenditure and glucose levels. For instance the brain contains neurons that sense glucose levels and responds by controlling peripheral tissues to react to changes in glucose levels.

The **pancreas** is the main peripheral organ involved in responding to changes in blood glucose levels by secreting insulin or glucagon. The organ is not only controlled by the brain but has intrinsic mechanisms of detecting glucose levels and responding appropriately. In addition to glucose, several hormones affect pancreatic hormone secretion.

The brain areas involved in sensing peripheral signals of energy status include the hypothalamus but also the nuclus tractus solitarius (NTS) located in the brainstem. Leptin is a peripheral signal that plays a vital role in satiety regulation acting on these neuronal circuits but, in addition, the hormone has an important function in the regulation of glucose homeostasis. In this study, we use mouse genetics to investigate the roles of leptin receptors in the endocrine pancreas as well as neurons of the NTS. The chief focus is the role of these receptors in pancreatic hormone secretion and the control of feeding and bodyweight. These are important areas, of considerable relevance to human health, since defects in the crosstalk between the signals from the periphery to the brain involving energy and glucose metabolism may predispose for obesity and type 2 diabetes mellitus.

Firstly, we investigate if leptin signalling in the pancreas has an effect on glucose homeostasis by affecting pancreatic hormone secretion.

Secondly, we explore leptin signalling in GLP1-expessing neurons and determine whether they can affect feeding via projections to the ARC or if they could affect glucose homeostasis by acting on the pancreas and modulating hormone secretion.

Thirdly, we also investigate the hypothalamic RIP2*Cre* neurons located in the ARC and their effect on satiety or the possibility that these neurons act on the pancreas to modulate pancreatic secretion.

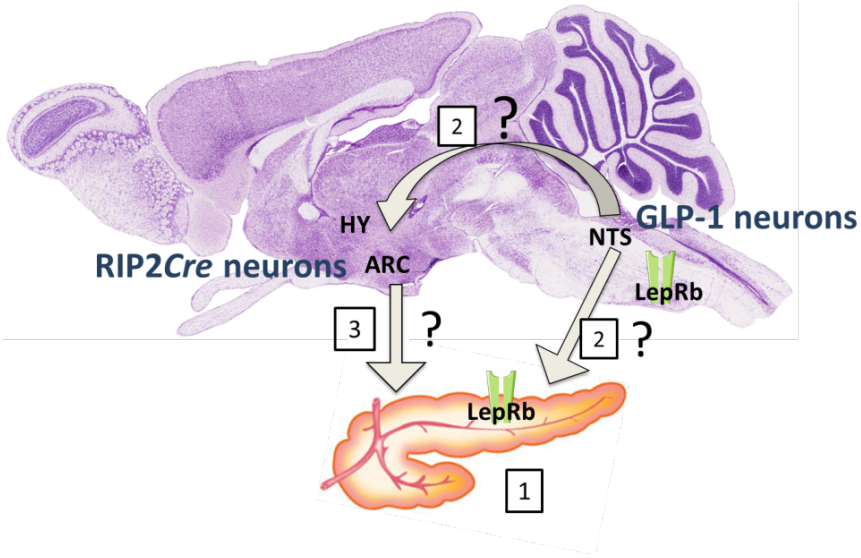


Figure 1.1 – Outline of projects. The figure is showing the pancreas, the brain with the location of ARC and NTS indicated. In project 1 we are interested in the LepRb in the pancreas and its effect on pancreatic hormone secretion. In project 2 we are looking at the LepRb in GLP-1 neurons of the NTS and their possible role in the regulation of pancreatic hormone secretion and a possible regulation of energy metabolism via the ARC. Lastly, we are also interested in the RIP2*Cre* neurons located in the ARC and their possibility to be involved in energy metabolism regulation. HY=hypothalamus, NTS= the nucleus of the solitary tract, LepRb=Leptin receptor**.**

1.1 Pancreas

1.1.2 The endocrine pancreas

The pancreas is involved in macronutrient digestion and energy homeostasis, secreting digestive and endocrine hormones. It is portioned into head, body and tail, located within the left abdominal cavity. The pancreas is both an exocrine and endocrine gland vital for nutrient metabolism and digestion [1]. The exocrine part contains mainly of acinar cells, producing digestive enzyme such as carboxypeptidase-A, amylase and trypsin, secreted via the pancreatic duct into the intestine to help the nutrient digestion of carbohydrates, lipids and proteins [2].

On the endocrine side, the pancreas secretes several hormones such as insulin, glucagon, somatostatin and pancreatic polypeptide, into the blood stream independently of the exocrine function. Cells secreting the hormones are clustered together forming the Islets of Langerhans and consist of α-, β-, δ-, γ- and ξ-cells [3].

1.1.3 Pancreatic development

During gestation, the pancreas is first morphologically apparent when the epithelial buds emerges. This occurs at embryonic day 26 in humans and on day 9 in mice [4]. This is followed by a process referred to as primary transition when the pancreatic buds elongates and branches out in tubular structures [5].

In the secondary transition, the number of endocrine cells expands, in particular the β-cells and rapid branching arises. The secondary transition starts at gestation day 37 in human and day 12 in mice and lasts until birth. During this stage the formation of rough endoplasmic reticulum, acinar enzyme expression exponentially increases and granules containing zymogen forms [1]. During the last stage the pancreatic cells differentiate and become fully specialized endocrine, acinar and ductal cells [3]

1.1.4 Islets of Langerhans

The islets of Langerhans, discovered by Paul Langerhans in 1869, are micro-organs inside the pancreas, containing five different types of endocrine cells. The clustered cells thus resembles “islands”. The islets of Langerhans occupy around 1-2% of the total volume of pancreas, or 1-2 mL of tissue in an adult human.

A few years after Paul Langerhans’ discovery, islets were found to contain different cell types, including α-cell and β-cells. The insulin secreting β-cells are the most abundant cells in pancreas representing 60-80% of the total islet [6]. The glucagon secreting α-cells represents 15-20% of the total islet [7]. Less abundant are

the δ-cells (3-10%), secreting somatostatin and γ-cells (3-5%), secreting pancreatic polypeptide (PPY) [6, 8]. The islets also contains ε-cells expressing the feeding hormone ghrelin [9].

The endocrine cells are not randomly distributed. In rodents β-cells situated in the core of the islets and non-β-cells including α-,δ-, and γ-cells are located in the mantle region [6, 7]. The arrangement in humans and non-human primates is less ordered, with α-cells (which are more abundant than in rodents, >40%) also present in the islet core [6].

Islets are highly vascularized, allowing for the cells to secrete their hormones into the blood stream [10]. Smaller islets receive capillaries that are integrated with the exocrine ones, whereas larger islets receive blood from one of three afferent arterioles [11].

1.2 Glucose stimulated insulin secretion

1.2.1 Pancreatic β-cells

The pancreatic β-cell secretes insulin in response to circulating plasma glucose levels, as well as to hormonal, nutritional and nervous stimuli, to ensure blood glucose levels are maintained at homeostatic levels (3-8 mmol/L). Secreted insulin reduces elevated glucose levels from the blood stream by acting on peripheral tissue promoting glucose uptake (skeletal muscle, adipose tissue) and by promoting the storage of glucose as glycogen (liver and muscle).

Each β-cell is poised to secrete insulin, harbouring 10,000-13,000 secretory granules [12], with around 7% docked at the plasma membrane and ready to secrete insulin [13]. The granules not only contain insulin but also around 50 polypeptides and low molecular weight compounds such as adenosine triphosphate (ATP), serotonin, γ-aminobutyric acid (GABA), glutamate and metal ions including Zn^{+} and Ca^{2+} [14]. These polypeptides include amyloid polypeptide (IAPP) with a relevant role in slowing gastric emptying [15] and chromogranin A involved in promoting the synthesis of secretory granules.

1.2.2 Insulin

Insulin was discovered by Banting and Best in 1921 and led to life-saving treatment of diabetic patients [16]. Several years later it was discovered that it is the pancreatic β-cells that produce and secrete insulin in response to glucose.

Insulin is transcribed and synthesized in β-cells into preproinsulin, a 110 amino acid insulin precursor [17]. Preproinsulin is further processed into proinsulin by removing its N-terminal signal peptide in the

endoplasmic reticulum. After protein folding and oxidation, proinsulin is transported by the Golgi apparatus and packaged into immature secretory granules. Insulin is processed from proinsulin by cleavage of a C-peptide intervening between the two peptide chains A and B. Mature insulin forms a hexamer with zinc, ultimately creating a crystalline lattice, and upon stimulation this is released alongside the C-peptide [18, 19]. Mature insulin can be stored in the secretory granules for several days or becomes degraded by lysosomes [20]

Mature insulin contains two peptide chains, chain A and chain B linked by two disulfide bonds and has a molecular mass of 6 kDa. The human insulin gene contains three exons and three introns [21]. The human insulin promoter is located within 300-400bp from insulins transcriptional start site containing several cis-regulatory elements [22].

In contrast to humans, rodents have two non-allelic genes of insulin, insulin I and insulin II (Ins1 and Ins2), where Ins2 is the human homologue [21, 23, 24]. The two forms of rodent insulin are very similar with 90% sequence homology, but the Ins1 gene only contains one of the two introns present in the Ins2 gene. At the protein level they only differ by three amino acids in the B chain and by two amino acids in the C-peptide. Ins2, in contrast to Ins1, is expressed in the developing brain [25, 26]. Ins1 expression is confined to the β-cells and is expressed at a lower degree with 1:2 ratios to Ins2 expression [25].

1.2.3 Glucose stimulated insulin secretion (GSIS)

Glucose-stimulated insulin secretion (GSIS) is the stimulus-dependent exocytosis of insulin from the β-cell. There are two processes how this is regulated. Firstly, it is suggested that receptors on the β-cell surface will be stimulated by both hormones and neurotransmitters or in second hypothesis where the metabolism of sugar will initiate the insulin secretion [27]. The latter hypothesis is believed to be the main stimulator of insulin secretion.

During a rise in blood glucose, glucose is taken up by β-cells via the glucose transporter isoform 2 (GLUT2). GLUT2 glucose transporter is expressed on the cell membrane of the β-cells [28]. GSIS is initiated by the rapid uptake of glucose and its subsequent phosphorylation by the low K_M (Michaelis constant) hexokinase glucokinase (GK) to form glucose-6-phosphate (G6-P) [29]. The formation of G6-P initiates glycolysis that converts G6-P into pyruvate in the cytoplasm. Pyruvate is subsequently taken up and oxidised by mitochondria resulting in the production of ATP [30]. Synthesized ATP is transported to cytosol via adenine nucleotide translocator that at the same time transports adenosine diphosphate ADP into the mitochondria [31].

The production of ATP leads to an increase in the ATP/ADP ratio that closes ATP-sensitive potassium ion channels (K_{ATP}),[32]. K_{ATP}-channels consist of four pore-forming subunits ($K_{ir}6.2$), surrounded by four regulatory subunits sulfonylurea receptor (SUR) [33]. The subunits are transcribed from the K^+ channel, Inwardly Rectifying Subfamily J, Member 11 (*KCNJ11*) and the (*SUR*) genes [34-36].

Intracellular ATP binds to the Kir6.2 subunit on the K_{ATP}-channels to cause channel closure [37]. K_{ATP}-channels regulate the membrane potential and the K_{ATP}-channels results in an ATP-mediated leak current. When K_{ATP}-channels current dominates, the cell membrane will hyperpolarize and thereby turning off electrical activity [38]. If on the other hand an inward current dominates (as in when the K_{ATP}-channels are almost completely closed) the membrane will depolarize. If this depolarization is sufficient an electrical activity will be initiated [38]. The membrane depolarization triggers an influx of Ca^{2+} as a result of the opening of voltage-sensitive, L-type Ca^{2+}-channels [39]. The influx and subsequent increase of cytosolic free Ca^{2+} ($[Ca^{2+}]_{cyt}$) initiates insulin exocytosis and is vital for significant insulin secretion [40].

There is some evidence that insulin granules form complexes with L-type Ca^{2+}-channels [41], favouring detection of microdomains of Ca^{2+} close to the mouths of these channels. Insulin exocytosis is also dependent on intracellular ATP for vesicles to fuse with the membrane and release their content. The fusion into the cell membrane is mediated by soluble-*N*-ethyl-maleimide sensitive factor (NSF) (SNARE) proteins. SNARE proteins thus mediate the docking of the insulin granules to the plasma membrane enabling exocytosis [42].

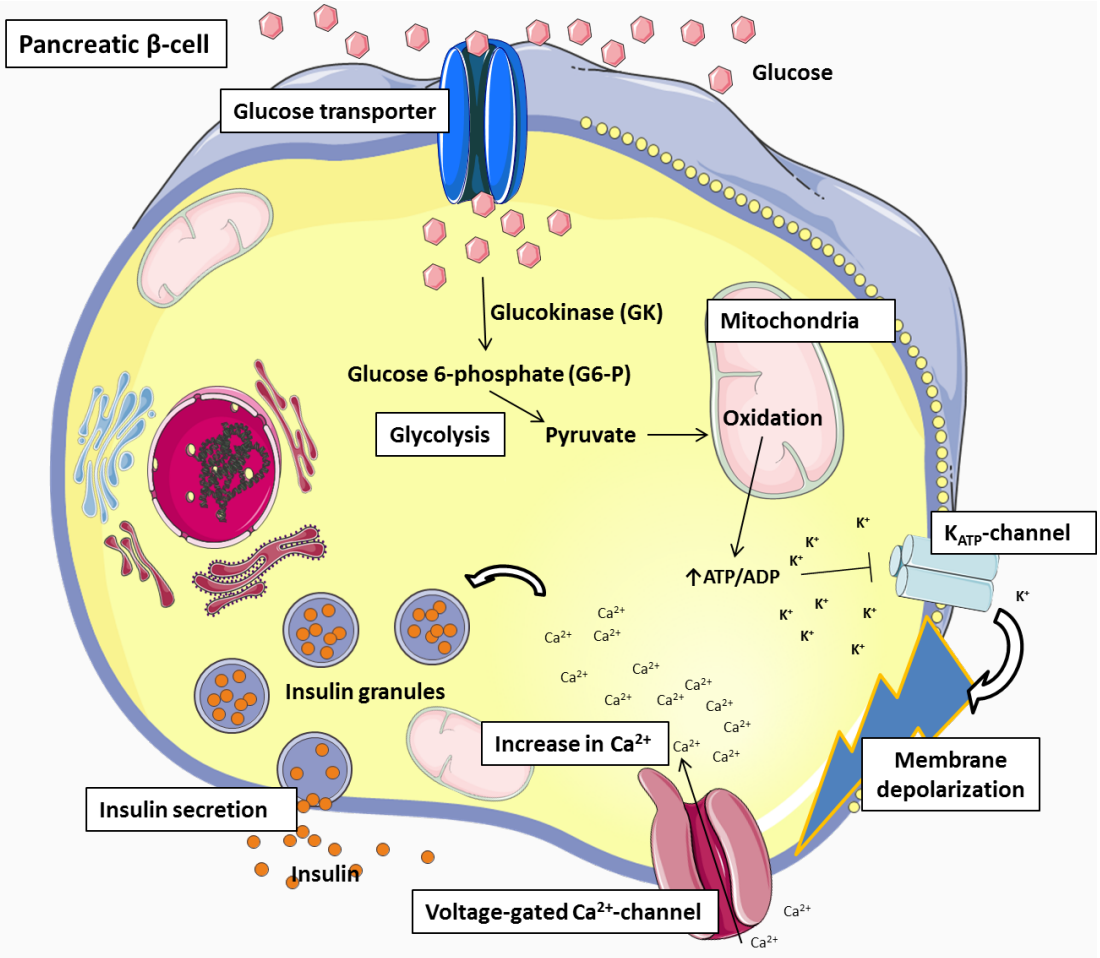


Figure 1.2 - Glucose uptake and subsequent insulin secretion. An increase in blood glucose levels leads to the uptake of glucose by the pancreatic β-cells via a glucose transporter. Inside the cell glucose is phosphorylated by glucokinase into glucose 6-phosphate (G6-P). G6-P is converted into pyruvate by glycolysis. The pyruvate enters the mitochondria where it is used to generate metabolic fuel in the form of adenosine triphosphate (ATP) via the tricarboxylic acid cycle (TCA-cycle). The production leads to an increase in ATP to ADP ratio that in turn closes the ATP sensitive K_{ATP}-channels. The closing of the K_{ATP}-channels results in a depolarization of the cell membrane, which in turn opens the voltage-gated Ca^{2+}-channels causing an influx of extracellular Ca^{2+}. The increase of extracellular Ca^{2+} in the cytosol triggers the insulin granules to secrete their insulin into the bloodstream via exocytosis. The figure is made from images from servier medical art.

1.2.4 The two-phases of insulin secretion

The glucose-stimulated release of insulin consists of two phases with the first phase delivering insulin transiently and rapid after a rise in blood glucose and lasts for a short period, approximately 2 min. Phase two is a prolonged and slow release of insulin lasting until the glucose levels are back to normal [43, 44].

The first phase requires a Ca^{2+} rise and the amplitude is directly affected by glucose induced $[Ca^{2+}]_{cyt}$ "amplifying pathway" [45]. The first phase of insulin secretion involves insulin-containing granules that are docked to the plasma membrane and are fused quickly releasing insulin to the blood stream. The second phase involves translocation of insulin granules and subsequent docking to the plasma membrane [46].

For the second phase insulin granules are synthesized and translocated, a step that is rate limiting in the exocytosis of insulin [47]. The second phase is, as the first-phase, triggered by the glucose induced rise in Ca^{2+} dependent and possibly additional signals [45, 48]. How the regulating mechanism in phase two insulin secretion functions remains uncertain in both human and rodent β-cells, but is mainly iterations of the first-phase insulin secretion [49].

1.2.5 Type 2 Diabetes mellitus (T2D)

Diabetes mellitus is characterized by elevated blood glucose levels (hyperglycemia). There are two main types of diabetes: type 1 diabetes (T1D) and type 2 diabetes (T2D). Type 1 diabetes is an autoimmune disease affecting 10-15% of diabetics. It is caused by the destruction of β-cells and thereby of a lack of insulin production in the body. In T2D the body cannot effectively respond to the insulin it produces due to insulin resistance and the failure of β-cells to compensate for the increased need of insulin.

Treatment for hyperglycemia is injection of insulin in T1D and also in later stages in T2D. Early stages of T2D are kept under control by diet and increasing exercise. The main cause of T2D is obesity, age but also genetic risk factors. Obesity as well as T2D are both linked to insulin resistance. However, not all obese with insulin resistance have hyperglycemia. Instead, their body releases more insulin and increases the β-cell mass to compensate for the increased need of insulin due to the reduced efficiency of insulin [50, 51]. For type 2 diabetes to occur, there must be an deficiency in releasing insulin [52].

1.2.5.1 Failure of the β–cell to compensate for insulin resistance

Type 2 diabetes usually occurs when the β-cells can no longer compensate for insulin resistance, though in many cases, and in particular in monogenic forms of the disease, β-cell defects are apparent early in disease development. A continued exposure to hyperglycemia results in a β-cell dysfunction [53]. Several molecular mechanisms have been proposed as to why this failure occurs. Exposure to chronic hyperglycemia (glucotoxicity) and elevated free fatty acids/ lipid intermediates (lipotoxicity) plays a central role in the mechanism in β-cell failure [54]. Increased levels of islet amyloid polypeptide (IAPP), inflammatory cytokines and genetic factors have also been proposed to contribute to β-cell failure [55].

1.2.5.2 Treatment of type 2 diabetes

Weight loss is a central goal for patients with T2D, in line with the fact that obesity is a main risk factor for T2D. Increased physical activity and weight loss is associated with improved glucose levels in T2D patients [56-58]. However, even though numerous research studies have shown the importance of lifestyle changes,

weight loss and exercise less than 50% of patients with T2D manage to achieve glucose control (glycated hemoglobin level <7.0%), [59].

More importantly, life style intervention has not decreased the cardiovascular effects in T2D patients. Therefore, pharmacology is necessary to treat type 2 diabetes targeting insulin resistance, insulin secretion, β-cell failure as well as obesity [60]. Several different drugs with different mechanism of action currently exist for the management of type 2 diabetes mellitus.

Metformin is a drug that is the mostly prescribed to treat T2D in children and adults to increase insulin sensitivity and thereby reduce hyperglycemia. The drug is proposed to suppress hepatic gluconeogenesis and improve peripheral glucose uptake [61]. The drug has also been shown to promote persistent weight loss [62].

Thiazolidenediones (pioglitazone) act by improving insulin sensitivity and restoring β-cell function acting on the peroxisome proliferator- activated receptors (PPARs), a group of nuclear receptors, they have the highest specificity is for PPARγ . Unfortunately, this drug also leads to a substantial weight gain and is therefore not always prescribed. Free fatty acids (FFA) and eicosanoids are the endogenous ligands for these receptors and when activated increase the storage of FFAs in adipose tissue. Increased storage of FFAs leads to less circulating FFAs resulting in cells becoming more dependent on the oxidation of carbohydrates in order to yield more energy. By reducing circulating fatty acid concentrations and lipid availability in liver and muscle, the drugs improve the patient's sensitivity to insulin. Thiaziolidiones reduce circulating lipids, ectopic fat in the liver and in the visceral department, but increases subcutaneous fat to increase insulin sensitivity and improves β-cell function. [63].

Sulfonylureas target the β-cell by binding the sulfonylurea receptor (SUR1) and thereby closing the K^{+}_{ATP}-channels which enhances insulin secretion [64]. Several types of sulfonyueras are available such as Tolbutamide, Glimepiride, Glipizide, Gliclazide and Glibenclamide. Patients treated with sulfonylureas are at risk for hypoglycaemia due to the enhancement of insulin secretion [65, 66].

Incretin analogues are analogues to the glucagon like peptide (GLP-1) that has been found to up-regulate glucose stimulated insulin secretion and reducing postprandial glucose levels [67]. Several receptor agonists of GLP-1 are currently approved such as exenatide and liraglutide. Treatment with GLP-1 analogues does not only improve glucose stimulated insulin secretion but also results in weight loss [68]. However, there are also concerns that the GLP-1 analogues are associated with increased risk for pancreatitis and pancreatic cancer.

Dipeptidyl-pepidase-IV (DPP-IV) is an enzyme that degrades GLP-1 in the blood stream [69]. Inhibitors to DPP-IV increase circulating GLP-1 levels and therefore have similar effect on glucose levels as the GLP-1 analogues.

Insulin is prescribed during later stages of T2D and to patients who are not responding well to insulin sensitizers. Patients treated with insulin are in the same way as sulfonylureas treated patients, at risk of hypoglycemia [70].

Several weight loss agents are being used and are in addition to affecting body weight also showing improved hyperglycemia. Phentermine-topiramate reduces weight and provides glycemic benefits. Phentermine acts on norepinephrine action in the hypothalamus to inhibit appetite and Topiramate inhibits lipogenesis and increases energy expenditure. Orlistat is an inhibitor that blocks fat uptake in the gastrointestinal tract and improves insulin sensitivity. Lorcaserin is an agonist of the serotonin 2C receptor and reduces appetite and food intake. Naltrexone-bupropion is combination tablet acting on the melanocortin system in hypothalamus and the mesolimbic reward system to inhibit food intake [71].

1.3 Pancreatic α-cells and glucagon secretion

Whilst insulin is secreted in response to feeding to lower glucose levels, glucagon is released in response to demand of glucose and is critical for glucose homeostasis. The hormone was discovered to be secreted from the pancreatic α-cells by Sutherland and colleagues [72] when circulating blood glucose levels falls below normal[73, 74]. Circulating glucagon act on to the glucagon receptor expressed on the liver, stimulating glucose output via glycogenolysis and gluconeogenesis and by decreasing glycogenesis and glycolysis.

Glucagon can, in small doses, rapidly and transiently induce an increase in blood glucose levels [75-77]. Impaired glucagon secretion is a factor contributing to the increased rate of glucose output and levels in T2D [78-80]. However, there are two aspects to the impaired secretion of glucagon in diabetes, one is an increased output during hyperglycemia and the second one is an impairment in the normal increase of glucagon during hypoglycemia in insulin treated-patients [81, 82].

1.3.1 Regulation of glucagon secretion from α-cells

Glucagon secretion is initiated when the blood glucose levels decrease to below resting blood glucose level of ~5mM. When blood glucose increases to 7-8mM glucagon secretion is inhibited [83]. The mechanism by which glucose regulates glucagon secretion is proposed to include the autonomic nervous system, paracrine effects and intrinsic glucose sensing mechanisms. The neuronal, intrinsic and paracrine mechanisms to control glucagon secretion are not mutually exclusive [84].

1.3.1.1 Regulation of glucagon secretion by autonomic nervous system

First, it is possible that glucagon secretion can be mediated by autonomic nervous system, by glucose levels being measured by sensors outside pancreas such as the portal vein sensors that contains vagal afferent that projects to the NTS, LHA and PVN. These neuronal pathways can in turn activate the α-cells to secrete glucagon via innervation of the sympathetic (adrenergic) and the parasympathetic (cholinergic nerves) [85, 86]. The parasympathetic nervous system also controls the counter-regulatory response. However, since the α-cells secrete glucagon upon isolation, this mechanism of regulation is not the only mechanism for glucagon secretion.

1.3.1.2 Regulation of glucagon secretion by paracrine release

Since α-cells secrete glucagon independently of neuronal connections, glucose sensing could be achieved by the neighbouring β- and δ-cells that control α-cells by paracrine release of insulin, Zn^{2+}, γ-aminobutyric acid or somatostatin. On the paracrine side, insulin has been shown to have an inhibitory effect [87, 88], by activating the K_{ATP} channels to hyperpolarize and close the voltage-dependent Ca^{2+} channels of the α-cells [89]. Insulin may also suppress glucagon secretion by activation of $GABA_A$ receptors expressed on the α-cell via an Akt-kinase dependent pathway that, when activated, hyperpolarizes the plasma membrane and thereby suppresses glucagon release [90].

In addition to insulin, the metal ion Zn^{2+}, which is co-secreted with insulin, has been suggested to have an inhibitory role in glucagon secretion [91, 92]. However, this view has been contested [93]. Somatostatin (SST) secreted by neural and endocrine cells such as pancreatic δ-cell is a potent inhibitor of glucagon and insulin secretion [94, 95]. The incretin glucagon-like peptide-1 (GLP-1 – see section 1.4, below) has been proposed to inhibit glucagon secretion [79], but the mechanism is not yet fully understood. Expression of GLP-1 receptors (GLP-1R) on α-cells was found to be vanishingly low [96] and therefore an effect of GLP-1 on glucagon secretion is most likely not directly via GLP-1R on α-cells. It has been proposed that GLP-1, by activating insulin and zinc secretion from the β-cells, inhibits glucagon release [89].

Glucagon itself has been demonstrated to amplify its own secretion by raising cAMP levels in α-cells [97, 98]. Glutamate that is co-released with glucagon can increase secretion by raising cytoplasmic Ca^{2+}. The satiety hormone leptin has been suggested to have an inhibitory effect on α-cells and glucagon secretion but at basal plasma leptin levels it was found to have a limited effect [99]. However, in an *in vivo* study an effect on glucose homeostasis after a partial deletion of leptin receptors in α-cells was found [100].

1.3.1.3 Regulation of glucagon secretion by intrinsic glucose sensing

In addition to the paracrine effect on glucagon secretion, evidence suggests α-cells possess an intrinsic glucose sensing mechanism affecting the secretion. Glucose transporter 1 (GLUT1) has been found to be expressed in rodents on α-cells but transport glucose into the cytosol 10 fold less than in β-cells [101]. The glucose sensor glucokinase (GK) that phosphorylates cytoplasmic glucose to form glucose-6-phosphate is expressed in α-cells similar to β-cells [102].

Both β- and α-cells are electrically excitable but the difference is that unlike β-cells α-cells fire action potentials [103-105] and has spontaneous oscillations of intracellular Ca^{2+} at low glucose concentrations (0–3 mmol/l) [106-108]. These spontaneous oscillations and electrical activity explains why glucagon is secreted during low glucose levels.

The actual mechanism through which glucagon secretion is inhibited at high glucose is contested. Studies have proposed that an increase in glucose levels hyperpolarises α-cells [109] and decrease the intracellular Ca^{2+} [110-113]. Other studies have demonstrated in isolated α-cells that they are electrically silent with low $[Ca^{2+}]_i$ levels at low glucose and an increase of glucose depolarise the α-cells and increase $[Ca^{2+}]_i$ [114]. In addition, there are reports from studies with intact islets that glucose has little effect on $[Ca^{2+}]_i$ levels [106, 115].

1.4 Glucagon-like peptide 1 (GLP-1)

GLP-1 is an incretin hormone released from the gut post-prandially. GLP-1 augments glucose-stimulated insulin secretion from pancreatic β-cells, inhibit glucagon release, delaying gastric emptying, decreases appetite and has a role in β-cell proliferation, differentiation as well as a decreasing β-cell apoptosis [116, 117].

Enteroendocrine L-cells in the ileum and colon sense nutrients such as luminal sugars, amino acids and fatty acids and secrete GLP-1 in response [118]. Administration of glucose orally triggers the GLP-1 secretion whereas no effect is seen if the intravenous route is used. After a meal GLP-1 is secreted into the blood stream and is rapidly inactivated by dipeptidyl-peptidase-IV (DPP-IV) [69] and has a half-life of around 2 min [119]. After secretion from the gut it acts on muscle, pancreas and liver to increase sensitivity to insulin, stimulation of GSIS and suppression of glucagon secretion. GLP-1 also has an effect locally on the intestinal wall to stimulate the enteroenteric reflexes that control gastric movements that in turn slow down the gastric emptying [117].

GLP-1 induces insulin secretion when blood glucose levels increases. However, when concentrations are below fasting blood glucose administration of GLP-1 fails to reduce blood glucose [120]. Because of GLP-1

benefits on glucose regulation GLP-1 analogues and inhibitors for DPP-4 are being used for the treatment of T2D [121]. The GLP-1 receptor (GLP-1R) has been identified in pancreas, adipose tissue, portal vein, lung, kidney, heart and vascular smooth muscles [96, 122] and in several areas of the brain [123]. Deletion of GLP-1r in rodents results in animal that have an impaired glucose tolerance and reduced glucose stimulated insulin secretion [124]. Impaired secretion of GLP-1 is a factor that might contribute to the development of obesity and T2D [125].

In addition to the production and secretion of GLP-1 from L-cells, GLP-1 is expressed in the CNS. GLP-1 expression and function in the brain is discussed in more detail in section 1.6.2.1.

1.4.1 GLP-1 signalling

GLP-1 exerts its effect by binding to the GLP-1 receptor (GLP-1R). The binding stimulates cyclic AMP formation and activation of downstream pathways coupled to protein kinase A and cAMP-regulated guanine nucleotide exchange factors [126]. GLP-1R activation promotes phosphorylation of cyclic AMP response element binding protein (CREB) in addition to regulating CREB activity via glucose–dependent stimulation of nuclear translocation of TORC2 a co-activator of CREB. The GLP1-R signalling also coupled to increased intracellular calcium and inhibition of voltage dependent K^+ currents activation of Erk1/2, protein kinase C, and phosphatidylinositol 3-kinase (PI3K) [117].

GLP-1 stimulates glucose-dependent insulin secretion by activation of its specific G protein-coupled receptors expressed on β-cells. The mechanisms by which GLP-1 stimulate insulin secretion only at elevated levels of plasma glucose is unclear. GLP-R activation stimulates cyclic AMP formation and activation of protein kinase A. The PKA-independent stimulation of insulin secretion by incretins has been attributed to guanine nucleotide exchange factors (GEFs), mainly cyclic AMP-GEFII (Epac2) a decrease of GEFII expression reduces the effects of GLP-1 on insulin secretion [127]. It has been proposed that the sulfonylurea receptor (SUR) subunit plays a role for in modulating GLP-1R-dependent K_{ATP} channels closure. Deletion of the SUR1 in mice markedly reduced the effect of GLP1 on insulin secretion [128, 129].

1.4.2 GLP-1 promotes β-cell proliferation and inhibition of apoptosis

GLP-1 has an effect on β-cell proliferation by affecting apoptosis. GLP-1 itself and GLP-1 analogues have been demonstrated to have anti-apoptotic properties in animal models [130, 131]. Mice deleted for the GLP-1R have an increased β-cell apoptosis and isolated islet are more susceptible to streptozotocin (STZ) treatment in comparison to control islets [131]. In human studies, islets incubated with GLP-1 have been found to be protected from glucolipotoxicity [132].

1.4.3 Proglucagon peptides and expression

The preproglucagon gene is transcribed to the primary transcript preproglucagon (PPG), which is then translated into proglucagon. Proglucagon is subject to tissue-specific posttranslational processing and formed, through the action of specific endoproteases, into GLP-1, GLP-2, glucagon, oxyntomodulin and glicentin depending on the tissue and cell type. In the endocrine pancreatic α-cells proglucagon is processed mainly into glucagon, whereas in enteroendocrine L-cells of the gut and in the brain is processed into GLP-1 together with GLP-2 [125].

The preproglucagon mRNA encodes a N-terminal signal peptide (SP), glicentin related pancreatic polypeptide (GRPP), unprocessed glucagon, unprocessed GLP-1 and GLP-2, separated by intervening protein 1 (IP1) or intervening protein 2 (IP2) as indicated in figure 1.3. The posttranslational processing processed differently depending on the presence of the prohormone convertase (PC) 1/3 and 2, now also referred to as PCSK1 and PCSK2 [125].

In pancreatic α-cells proglucagon is processed mainly into glucagon due to the presence of abundant PC2 (PCSK2) [133]. By contrast, in enteroendocrine L-cells of the gut as well as in several brain regions the same proglucagon molecule is processed into GLP-1 together with GLP-2 and oxyntomodulin, due to the presence of the PC1/3 (PCSK1) [133, 134]. PC1/3 expression has been detected in islets as well as traces of active GLP-1 suggesting a possible role of for GLP-1 [135, 136]. PC2 has also been detected in the brain but has not been co-localized to expression of PPG, suggesting that PC1/3 is predominant in the brain [134].

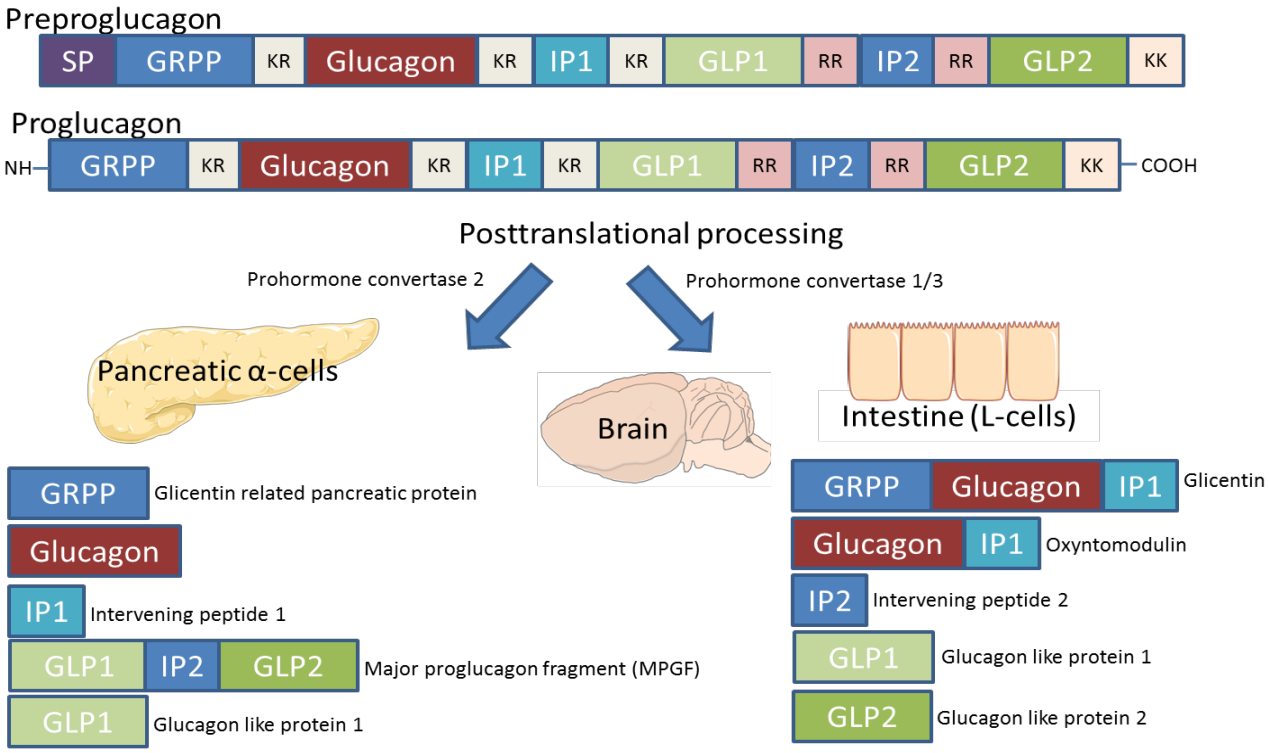


Figure 1.3 - Schematic illustration of preproglucagon postranslationøl processing. Preproglucagon (top) consist of n-terminal SP, GRPP, glucagon, GLP-1, GLP-2 separated by intervening proteins 1 and 2. KR, RR. KK denotes dibasic amino acids residues that are potential sites for cleavage by the prohormone convertases (PC). Preproglucagon encodes proglucagon, that is via posttranslational processing with either PC2 or PC1/3 processed into different peptides. In α-cells PC2 predominates and proglucagon is processed into GRPP, glucagon, IP1, major proglucagon fragment (MPGF) and possibly GLP-1. In the brain and intestine PC1/3 is expressed and proglucagon is processed into glicentin, oxyntomodulin, IP2, GLP-1 and GLP-2. The figure is made from images from servier medical art.

1.5 The satiety hormone leptin

The *ob* gene product leptin encodes an adipose tissue-derived hormone that plays an important role in suppressing appetite and acts on neuronal circuits involved in energy homeostasis [137]. Before it was identified and cloned in 1994 [138], its existence was predicted by Douglas Coleman at the Jackson laboratories. Thus, Coleman and colleagues studied *ob/ob* mice (leptin deficient) and *db/db* mice (leptin receptor deficient) and from their "parabiosis" studies (in which the circulation of two mice is shared after surgery) they concluded that *ob/ob* mice were missing a circulating factor that was present in high concentrations in the *db/db* mice. Injection of this circulating factor would cure *ob/ob* mice but not *db/db* mice [139].

After its discovery, it was initially hoped to be the cure for human obesity and injection of leptin managed to correct the obesity caused by the rare cases of leptin deficiency in humans [140] and in rodents [141]. Unfortunately, most obese rodents are unresponsive to leptin and injections does not work on reducing body

weight and/or appetite [142]. Same goes for the majority of obese and overweight patients, a condition now referred to as leptin resistance [143]. This correlates with the findings that most obese patient have high circulating levels of leptin that correlates with their amount of adipose tissue [142].

Several different mutations of the leptin gene have been discovered in humans and cause severe early onset obesity. Currently, 11 different mutations have been described [144]. Leptin-deficient humans have been treated successfully with leptin replacement therapy [145]. Mutations in the leptin receptor in humans have also been found to lead to morbid obesity. Both leptin and leptin receptor deficiency leads to rapid weight gain during the first few months of life and hyperphagia [144].

1.5.1 Structure, production and expression of leptin

The leptin gene is located on chromosome 7 and transcribes into a 167 amino acid peptide. The amino acid sequence is 84% identical between mouse and humans [138]. There are leptin orthologues in amphibians, fish and reptiles. Although the adipose tissue is the main source for leptin production it has been found to be produced by peripheral tissues, such as the skeletal muscle, liver [146], the placenta and ovaries [147, 148].

The amount of leptin is secreted in correlation to the amount of adipose tissue the body contains [149]. Leptin levels decrease during fasting, which results in an increase in appetite [137], whereas feeding and obesity increases levels [150]. The decrease of leptin levels during fasting occurs rapidly and precedes the secretion correlated to the amount of adipose tissue [151]. Leptin is also secreted differently during the day with highest levels of circulating leptin present in the evenings [152].

1.5.2 Leptin signalling

Leptin mediates its effects by interaction with leptin receptors (LepR also called OB-R), which are highly expressed in the hypothalamus and to a lesser extent in other brain areas. Leptin is secreted in the periphery and reaches the CNS via the blood brain barrier (BBB), by receptor mediated endocytosis [153].

There are six isoforms of LepR (a-f) created by alternative splicing. The isoforms share the extracellular leptin binding domain but differ in length of the intracellular domain. The receptor contains an extracellular ligand-binding domain, a transmembrane domain and a intracellular signalling domain [154]. The longest isoform LepRb contains the intracellular signalling domain Janus kinase (JAK) binding domain box 1 and box 2 [143]. The leptin receptor is a class I cytokine receptor. These receptors signal via cytoplasmic tyrosine kinases belonging to the Janus kinase family (JAKs) and signal transducers and activators (STAT). Cytokine receptor signalling requires Box 1 and Box 2 motifs that are essential for JAKs kinase interaction

and activation. Only the longer isoform LepRb contains Box 2 and is therefore the only form that can signal. This is consistent with the lack of Box 2 in *db/db* mice that have a non-functional leptin receptor [155].

When leptin bind its receptor a dimerization occurs and the long form of the receptor activates the kinase JAK2 that phosphorylates itself and three tyrosine sites on the intracellular domain of the receptor (Y985, Y1077 and Y1138), figure 1.4 [156]. The auto-phosphorylation of JAK2 allows for the binding of the adapter protein Src homology 2 B 1 (SH2B1), the binding enhances JAK2 activation and helps recruits insulin receptor substrate 1 (IRS1) and insulin receptor substrate 2 (IRS2) to the complex [157]. This is followed by JAK2-mediated tyrosine phosphorylation of IRS1/2 and thereby activating the phosphoinositide 3-kinase pathway.

The phosphorylation on Y1138 results in the recruitment of STAT3 and subsequent phosphorylation on Y705 on STAT3. The phosphorylation causes STAT3 to dimerize and translocate to the nucleus to activate transcription to mediate its effect on energy homeostasis. [143]. In addition, one of the STAT3 activated genes is SOCS3, a feedback inhibitor [158].

Y985 phosphorylation recruits protein tyrosine phosphatase to the receptor that activates extracellular signal-regulated kinase (ERK)-signalling pathway. Y985 also contains a binding site for SOCS3 and thereby plays a role in the negative feedback inhibition of the leptin signalling [158]. This is consistent with mice that lack the Y985 site are lean instead of obese due to the animals deficiency in negative feedback inhibition [159].

Phosphorylation of Y1077 and possibly Y1138 activates STAT5 that mediates leptin reproductive involvement [156]. Deletion of the Y1077 site in mice has a small impact on food intake [160]. The leptin dependent STAT3 phosphorylation (pSTAT3) is being used as a reliable marker for identification of leptin responsive cells [161, 162].

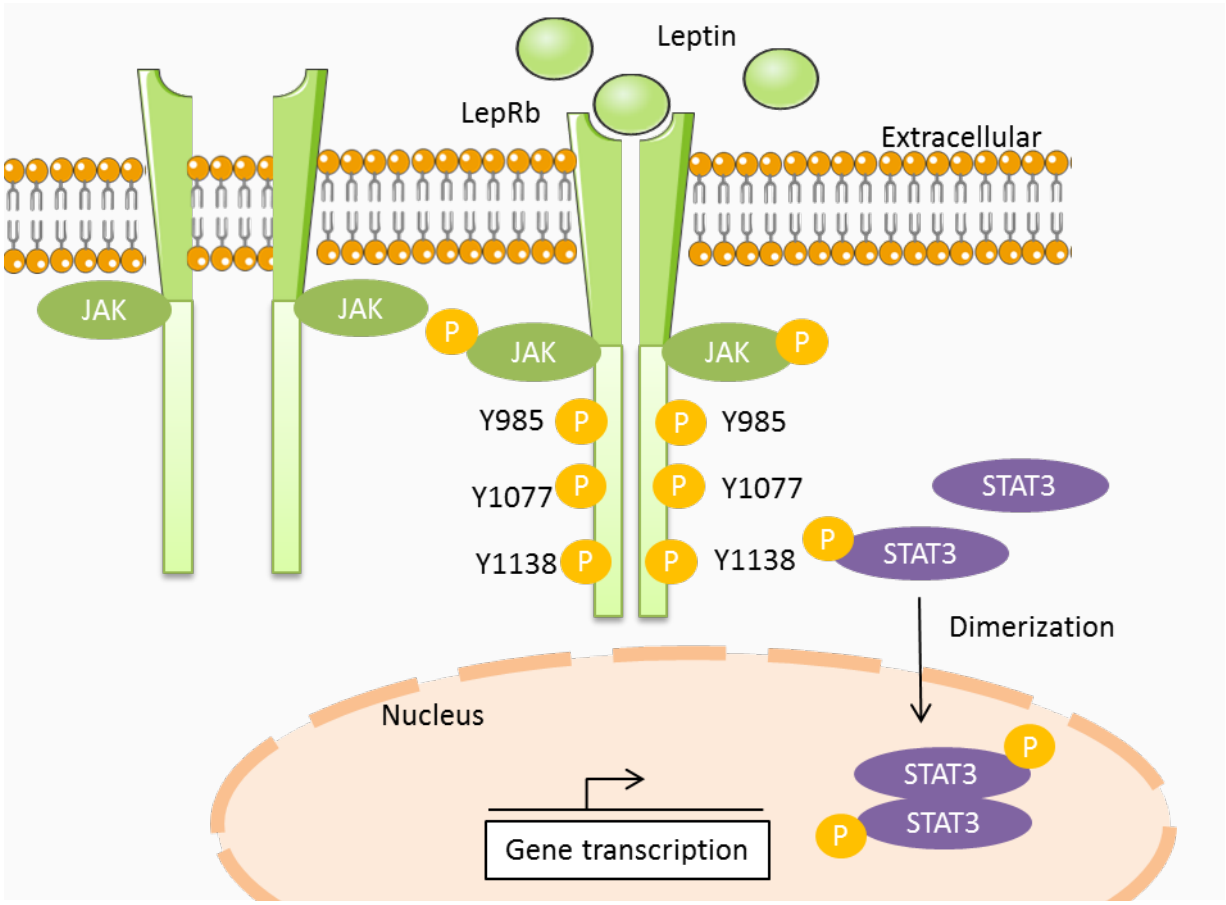


Figure 1.4 - Leptin signalling. Leptin binding to its receptor causes the receptor to dimerization and activate the associated JAK-2 tyrosine kinase pathway. The JAK-2 tyrosine kinase pathway phosphorylates itself on three intracellular sites activating different pathways. Y1138 mediates phosphorylation by JAK-2 of STAT3, which dimerizes and translocate to the nucleus and activates gene transcription. The figure is generated from images from servier medical art.

1.5.3 Leptin receptor function in the brain

Several different studies have deleted the leptin receptor (LepRb) in neurons. A total ablation of all receptors in the CNS results in animals that are suffering from obesity, mimicking the effect of leptin deficient mice *ob/ob* and the leptin receptor deficient mice *db/db* [163]. In addition, several studies with selective deletions in specific neurons have been found to have an effect on energy balance. Selective deletion of the leptin receptor in the pro-opiomelanocortin (POMC) neurons in mice leads to animals that have a mild obese phenotype and hyperleptinaemia [164]. In another research study they ablated the LepRb in mice in AgRP neurons and found that mutant mice had an increase in body weight [165]. Dhillion and colleagues deleted the leptin receptor in steroidogenic factor 1 (SF1) neurons located in the VMH of hypothalamus resulting in animals that had a modest obesity [166].

These research results cannot fully reproduce the obese phenotype observed in *db/db* mice suggesting that there are other neurons expressing leptin receptors that contribute to leptin's role in regulation of feeding and body weight.

1.5.4 Leptin's effect on glucose homeostasis

Apart from a role in satiety, leptin has been shown to have an effect on glucose metabolism unrelated to its role in energy balance. It was first observed that *ob/ob* mice and *db/db* mice apart from their obesity were hyperglycemic, hyperinsulinemic and insulin resistant, similar to type 2 diabetes [167, 168]. Further supporting the independence of body weight in leptin's role in glucose homeostasis is a study when injection of leptin in *ob/ob* animals improved hyperglycemia and hyperinsulinemia before an effect on body weight was observed [169], demonstrating the effect on glucose homeostasis by leptin precedes the change in body weight and obesity. Also supporting this hypothesis is a study where leptin signalling was disrupted by injecting a leptin antagonist in to wild-type mice. Mice administered with the leptin antagonist exhibited fasting and glucose stimulated hyperinsulinemia and became insulin resistant prior to a significant change in body composition and increase in body weight [170]. This is further supported by rodents and humans who have a complete lack of adipose tissue (lipodystrophy) and are thereby hypoleptinemic and have hyperinsulinemia, insulin resistance and hyperglycemia [171, 172]. Irrespective of the amount of adipose tissue abnormally low leptin levels lead to defects in glucose homeostasis, further supporting leptin's effect on glucose homeostasis is not secondary to the obesity.

With several studies demonstrating an effect of leptin on glucose homeostasis, it remains to be determined what specific target tissues mediate leptin's action on glucose homeostasis. To mediate its effect leptin could act either via centrally expressed leptin receptors and/or via peripherally expressed leptin receptors. Insulin secretion in leptin treated islets has been investigated in several studies but with very opposing results. The question is whether leptin affects insulin secretion? If so, is this by acting directly on the β-cells, via central actions and/or via other peripheral tissues?

1.5.4.1 Leptin's effect on insulin secretion in isolated islets

Several research groups have investigated insulin secretion from isolated islets, isolated perfused pancreas or insulin producing cell lines but with no clear answers to whether leptin act directly onto β-cells to modify insulin secretion. To interpret these studies, you would have to take into account that different recombinant leptin, leptin concentrations, different animal models and different external stimulus have been used. The majority of groups can see an inhibition of insulin secretion; however, this is observed at different concentration of glucose (glucose stimulating insulin concentrations versus basal glucose concentrations).

Kieffer and colleagues [173] were the first to demonstrate that leptin receptor was expressed in pancreatic β-cells. This discovery was followed by Emilsson and colleagues [174] who demonstrated that leptin affected insulin secretion in isolated islet. They observed an inhibition of insulin secretion at supraphysiological concentration leptin in perfused pancreas from *ob/ob* mice at basal glucose concentration. No effect was observed on perfused pancreas from Zucker *fa/fa* (leptin receptor deficient) rats, suggesting that the leptin receptor is needed for the inhibition of insulin. In addition they also demonstrated an inhibitory effect at supraphysiological leptin concentrations at this time at glucose stimulating insulin secretion (GSIS) concentrations and in isolated islets. Again this effect was lost in islets from *db/db* mice suggesting the LepRb is required for the effect.

Kieffer and colleagues [175] further characterized this by demonstrating that leptin inhibited insulin secretion at both basal and high glucose concentrations using isolated islets from *ob/ob* mice. In addition to Kieffer and colleagues, a few more studies found leptin to have an inhibitory effect on insulin secretion, at high glucose levels only [176] or at basal glucose levels only in rodent [177] and human [178]. Fehmann and colleagues observed an inhibition at both high glucose levels and basal glucose levels. In a second set of experiments they observed after the addition of leptin the Ca^{2+} oscillation amplitude decreased as well increasing the frequency.

Stand alone is a research study by Kulkarni and colleagues which is the only study to demonstrate an inhibition of insulin secretion in the range of normal circulating leptin concentration. They observed an inhibition in both rat islets and human islets at high glucose concentrations only [179].

Another group, investigated isolated islets from rat exposed to circulating levels of leptin (0.7nM) but failed to see an inhibition neither at low glucose levels nor high glucose levels of leptin [180]. However, this group did find inhibition after addition of the insulin release stimulator 3-isobutyl-1-methylxanthine (IBMX) in combination with high glucose but the effect was lost at low glucose levels. This effect was lost in the obese Zucker *fa/fa*. Their study suggests that leptin inhibition is not via the ATP-sensitive potassium channels as proposed by Kieffer and colleagues [175].

Even more inconsistently with the previous research studies, are three different research groups that observed an enhancement of insulin secretion after exposing cell lines MIN6 and HIT-T15 to leptin [181, 182]. Tanizawa and colleagues also found that leptin (1nM) enhanced insulin secretion in isolated islets from rat at basal glucose levels that changed to a small inhibitory effect at high glucose levels. Suggesting that leptin stimulates insulin secretion during normal conditions but at glucose stimulating insulin secretion concentrations it turns to inhibition of insulin secretion.

An stimulatory effect of insulin secretion by leptin is also observed by Ceddia and colleagues [183] in isolated islets from rat at basal glucose levels. During the addition of high glucose the leptin treated islet did not present a first-phase insulin secretion response but eventually reached the same values such as the control group. This is similar to Roduit and colleagues [184] study where they saw an inhibition at first phase response, however, only after 6 hrs incubation with leptin.

A few groups have also failed to see any effect of leptin on insulin secretion. Leclercq-Meyer and colleagues used isolated perfused rat pancreas using 1nM of human leptin and high glucose and saw no effect on insulin secretion [185]. This result was again confirmed by a ten times higher concentration of leptin [186]. In a third study from the group [187], they confirmed their previous findings with exposing isolated perfused rat pancreas with a differently purified human and murine leptin at the concentration of 10nM in the presence of basal glucose and high glucose again found no effect of leptin on insulin secretion. They also investigated leptins effect on glucagon and somatostatin secretion but failed to see an effect. In a similar study by Rodriguez-Gallardo *et al*. 1997 (reported in abstract form at IDF, Helsinki), no effect of leptin was found in perfused pancreas at leptin concentrations from and exposure to rising glucose concentrations.

It is important to note that the majority of studies where they found an inhibitory effect on insulin secretion are at supraphysiological concentrations; normal plasma leptin concentration is in the range of 0.06-0.6nM [188]. There is a possibility that leptin could be toxic to islets at these high concentrations. Secondly, it is important to notice that experiments carried out in *ob/ob* mice with a defective leptin are probably not representative of the effect of leptin on normal animals considering they have never been exposed to leptin and *ob/ob* islets are very sensitive to the hormone. Interestingly, studies that observed an effect on insulin secretion upon leptin treatment made the observation under different glucose concentrations i.e. at high glucose levels where insulin secretion is stimulated or at basal glucose levels. All the conditions, leptin concentration, effect on insulin secretion and model are listed in table 1.1.

Table 1.1 Leptins effect on insulin secretion

Condition	Leptin	Effect on insulin secretion	Model	Ref.
1. Basal 2. HG (16.7mM)	100nM 10-100nM	Inhibition Inhibition	Perfused pancreas, mice islets	[174]
1. Basal 2. HG (11.1mM)	6.2nM	Inhibition	Mice islets (Ob)	[175]
1. HG (10mM-20mM) 2. HG (10mM) +10μM acetylcholine 3. HG (10mM) +0.1μM PMA	1. 20 -100nM 2. 20nM 3. 20nM	1. 0 2. Inhibition in OB (not in WT) 3. Inhibition in OB (not in WT)	Mice islets (Ob and lean)	[189]
1. Basal (3.3mM) 2. HG (8.3mM)	1nM	1. 0 2. 0	Rat islets	[185]
1. 3.3mM glucose 2. 8.3mM glucose	10nM (human+mouse)	1. 0 2. 0	Rat islets	[187]
1. 2.8-16.7mM glucose 2. 2.8-16.7mM glucose 3. 2.8-16.7mM glucose	1.10-100nM 2.1nM (24 hrs) 3.10-100nM (24 hrs)	1. 0 2. 0 4. Inhibition	Rat islets	[184]
1. Basal 2. HG (16.7mM) 3. HG (16.7mM) +0.1mM IBMX	0.6nM	1. 0 2. 0 3. Inhibition	Rat and mice islets	[180]
7mM glucose	1a. 0.065nM 1b. 0.65nM 1c. 6.25nM (20 min) 2a. 0.065nM 2b. 0.65nM 2c. 6.25nM (2 hrs) 3a. 0.065nM 3b. 0.65nM 3c. 6.25nM (24 hrs)	1a. 0 1b. 0 1c. Stimulation 2a. 0 2b. 0 2c. Stimulation 3a. Stimulation 3b. Stimulation 3c. Stimulation	HIT-5	[181]
1a. Basal 1b. Basal 2. HG (25mM) 3. HG (16.7mM)	1a. 1nM (30min) 1b. 1nM (2hr) 2. 1nM 3. 10-100nM	1a. 0 1a. Stimulation 2. 0 3. Inhibition	MIN6, Rat islets	[182]
1. LG (2.8mM) 2. Basal 3. HG (16.7mM)	50nM	1. Stimulation 2. Stimulation 3. 0	Rat islets	[183]
1. Basal 2. HG (11.1mM)	1. 80nM 2. 80nM	1. Inhibition 2. 0	Rat islets	[177]
1. Basal 2. HG (10mM)	1-10nM	Inhibition	Perfused rat pancreas, mice islets, INS-1 cells	[190]

1. Basal (5.6mM) 2. HG (11.1)	6.25nM	1. Inhibition 2. 0	Human islets	[178]
1. LG (2.8mM) 2. Basal 3. 8mM glucose	0.1nM 10nM	1. 0 2. Inhibition 3. Inhibition	Insulinoma cells, rat and human islets and, *in vivo*, in mice.	[179]
1. HG (20mM) 2. 4-10mM glucose 3. HG (16-20mM) 4. HG (24mM)	1a. 100nM 1b. 10nM 1c. 1nM 1d. 0.1nM 2. 10nM 3. 10nM 4. 10nM	1a. 0 1b. Inhibition 1c. Inhibition 1d. 0 2. 0 3. Inhibition 4. 0	Rat Islets	[176]
1. HG (8 mM)	1. 10nM	1. Inhibition	Mouse islets	[191]

HG = high glucose, basal= 5.6mM, LG=low glucose

1.5.4.2 Leptin's effect on insulin secretion in *in vivo* models

With rather inconclusive results for leptin's effect *in vitro* on insulin secretion a few groups have disrupted leptin signalling in β-cells using transgenic mice models, as shown in table 1.2. In one study, they deleted the long form of leptin receptor in β-cells using the rat insulin 2 promoter *Cre* (RIP2*Cre*) driver [192] (a description of the Cre*Lox*P system for deleting genes tissue-specifically is provided later in Section 1.8). The RIP2*Cre* is active in specific neurons (RIP2*Cre* neurons) in hypothalamus [193] as well as in the pancreatic β-cells, thereby disrupting leptin signalling in both these two areas. These animals are obese, hyperinsulinemic and with impaired glucose-stimulated insulin release and glucose intolerance.

On the other hand, in a study by Morioka and colleagues [191], the authors deleted the leptin receptor in all pancreatic cells using a pancreas duodenal homeobox 1 (Pdx1) promoter driving *Cre* recombinase. In contrast, these animals had a normal bodyweight and an improved glucose tolerance but consistency with the RIP2*Cre*LepRb^{KO} they were hyperinsulinemic.

In a third study, Guo and colleagues deleted all peripheral leptin receptors including pancreatic leptin receptors but none in the brain [194]. Surprisingly, this group found no effect on glucose homeostasis but their animals were hyperleptinemic.

Table 1.2 *In vivo* models of LepRb^{KO}			
Model	**Deletion**	**Effect**	**Ref.**
RIP2*Cre*.LepRb^{KO}	β-cells and brain	Obesity and impaired glucose tolerance	[192]
PDX1*Cre*.LepRb^{KO}	Pancreatic cells and brain	Improved glucose tolerance	[191]
ROSA26*Cre*^{ERT2}.LepRb^{KO}	Peripheral	Normal glucose tolerance but hyperleptinemia	[194]

These are two rather similar animal models both deleting the leptin receptor in pancreatic β-cells but with very opposing phenotypes, listed in Table 1.3. The difference in phenotype is most likely due to the difference in transgenic animal used to delete LepRb.

Morioka and colleagues [191] used a transgenic mouse with a floxed allele that upon *Cre* expression removes the first exon and thereby all isoforms causing a total ablation of the leptin receptor. Covey and colleagues [192] used a transgenic mouse that has exon 17 floxed [195], resulting in expression of the LepRb but a truncated form of the receptor that lacks both Y985 and Y1138, necessary for JAK-mediated phosphorylation and leptin signalling.

The difference in these two studies could also be due to the two different *Cre* drivers, that delete in overlapping but different tissues, none exclusively in the β-cells. The RIP2*Cre* promoter used by Covey and colleagues is a commonly used *Cre* driver that generates β-cell deletions but, in addition, it deletes in RIP2*Cre* neurons expressed in brain areas involved in glucose homeostasis and feeding regulation [193].

In contrast, *the* Pdx1Cre promoter [196] used by *Morioka and colleagues* [191], is expressed during development in the posterior foregut and thereby deletes in cells that secretes glucagon, GLP-1, GIP, Ghrelin and others with known effects in glucose homeostasis [197]. Furthermore, the Pdx1*Cre* promoter is expressed in the hypothalamus of the brain as demonstrated in figure 1.5A [193]. In addition, it has also been *proven by staining for p*STAT3 an indicator for leptin signalling by Wickstead and colleagues that the Pdx1*Cre* is expressed in leptin responsive neurons located lateral hypothalamus (Figure 1.5 C-H) [193].

Surprisingly, when the animals deleted for LepRb with the Pdx1*Cre*-promoter were challenged with a high fat diet their glucose tolerance changed from improved to impaired glucose tolerance as well as an impaired glucose stimulated insulin secretion. It is possible that the animals with RIP2*Cre* driver have an impaired glucose homeostasis due to their obesity and thereby an impaired β-cell function. Indeed, the same results were obtained when the Pdx1*Cre* mouse was put on a high fat diet. This suggests that animals that lack β-cell signalling are sensitive to obesity by developing impaired β-cell function. The two different studies just reached obesity by two different means: genetically engineered or high fat diet.

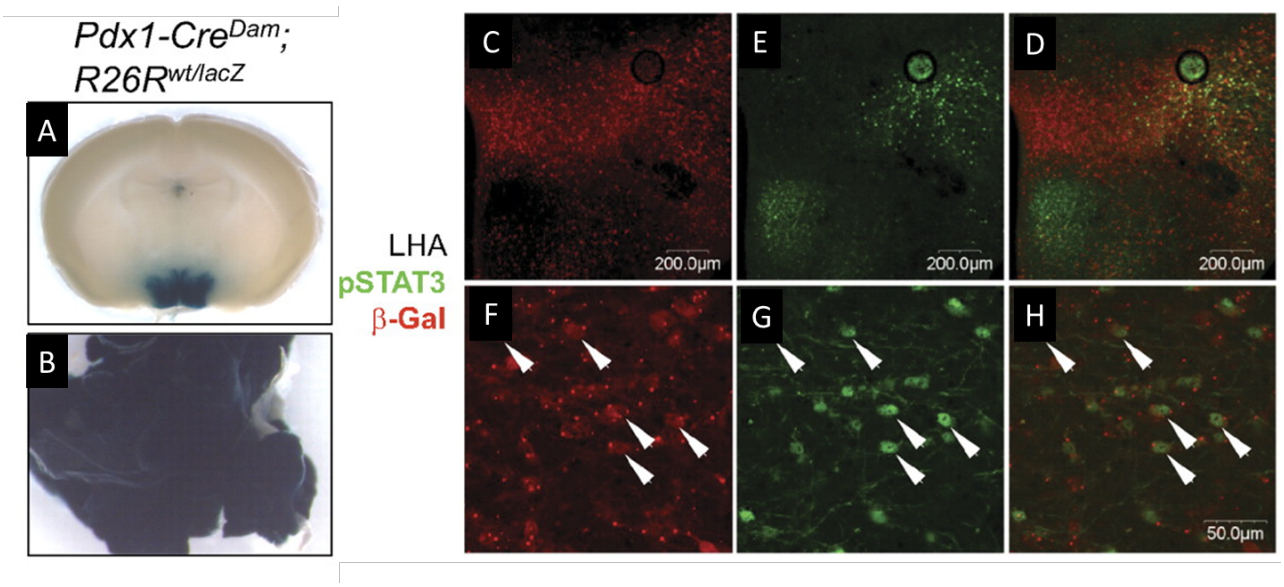


Figure 1.5. Cre-mediated recombination in Pdx1Cre transgenic animals visualized with a R26R^{LacZ} Cre reporter strain. A: Pdx1Cre expression was localized to hypothalamus. B: Pdx1Cre-mediated recombination in pancreas. C-H showing staining for leptin signalling (pSTAT3, green) and *Cre*-mediated recombination (*β-gal, red)* in the lateral hypothalamus. Arrows are pointing at co-localisations between leptin responsive and Pdx1*Cre* mediated recombination in a subpopulation of neurons. Image was adapted from Wickstead and colleagues [193].

However, the findings of a third study, by Guo and colleagues [194], are particularly important in this context: this involved deletion of all peripheral leptin receptors but keeping the ones in the brain intact. This study revealed no effects on glucose tolerance, suggesting that it is the central actions of leptin that are responsible for the phenotypes observed by Morikas' and Covey's studies.

Table 1.3 Phenotype of mice deleted for pancreatic LepRb

RIP2*Cre*.LepRb^{KO}	**PDX1*Cre*.LepRb^{KO}**	**PDX1*Cre*.LepRb^{KO} on high fat diet**	**ROSA26*Cre*^{ERT2}.LepR^{KO}**
Obese	Normal body weight	Normal body weight	Normal body weight
Impaired GT	Improved GT	Impaired GT	Normal GT
Impaired GSIS	Enhanced insulin secretion	Impaired GSIS	n/a
Insulin resistant	Normal	Normal	n/a
n/a	Enhanced intracellular Ca^{2+}	n/a	n/a
Increased islet size and density	Increased β-cell mass	Reduced islet size	n/a
Fasting hyperinsulinemia	Fasting hyperinsulinemia	n/a	n/a
Fasting hypoglycemia	Normal	n/a	n/a
Increased leptin levels	Normal	n/a	Increased leptin levels

1.5.5 Leptin receptor expression and localization

1.5.5.1 Leptin receptor expression in the brain

The leptin receptor is expressed in most parts of the brain and is particularly abundant in the hypothalamus. Several studies have identified LepRb mRNA expression in the mouse and rat brain via *in situ* hybridization. Expression was observed in the arcuate, ventromedial, paraventricular, periventricular and ventral premammilliary nuclei of hypothalamus [198, 199].

Expression has also been identified outside of the hypothalamus in areas such as the hippocampus, piriform cortex geniculate nuclei, several thalamic nuclei, substantia nigra pars compacta, nucleus of the lateral olfactory tract, and medial habenular nuclei in the mouse [199], rat [198] and human brain [200]. Expression has been reported in the hindbrain in mouse and rat with strong hybridization signals in the nucleus tractus solitaries (NTS) [201]. These finding are consistent with the examination of the product of the early gene, c-fos after stimulation with leptin [202-204].

The distribution of the leptin receptors in the brain has also been investigated with transgenic mice with the leptin receptor promoter driving *Cre* and in addition to a *Cre*-inducible enhanced green fluorescent protein (eGFP) [205]. This group further analysed the neurons expressing the receptor by utilizing a transgenic mouse harbouring a farnesylated enhanced green fluorescent protein (EGFPf) that locates eGFP to the membrane, enabling labelling of axonal projections.

The greatest number and density of LepRb-positive neurons was found in hypothalamus including the greatest density of projections. The strongest expression of LepRb neurons in hypothalamus was in the arcuate nucleus, dorsomedial hypothalamus and ventral premammillary (PMv) nucleus (Table 1.4). Projections in the ARC were distributed throughout whereas in the PMv they were located in the middle.

Table 1.4 Leptin receptor expression and localization in the brain		
Forebrain	**LepRb**	**Projections**
Claustrum	++	+++
Somatosensory cortex	+++	+
Auditory cortex	+++	++
Ectorhinal cortex	+++	+
Dorsal endopiriform nucleus	+++	+
Ventral endopiriform nucleus	+++	+++
Dentate gyrus	+	+++
Central amygdala	+	+++
Arcuate nucleus	++++	++++
Dorsomedial hypothalamus	++++	+++
Lateral hypothalamus	+++	++
Paraventricular nucleus	+	+++
Premammillary nucleus, ventral (PMv)	++++	++++
Ventromedial hypothalamus	+++	++
Median eminence	+/−	++++
Posterior hypothalamus	+++	++
Preoptic nuclei	+++	+++
Midbrain	**LepRb**	**Projections**
Commissure of inferior colliculus	+++	+
Ventral tegmental area	+++	+++
Substantia nigra	+++	+++
Edinger–Westpal nucleus	+++	+++
Linear raphe	++	+++
Dorsal raphe	+++	++++
Periaqueductal gray	++++	++++
Interfascicular nucleus	+	+++
Deep mesencephalic nucleus	+++	++
Hindbrain	**LepRb**	**Projections**
Nucleus of the solitary tract	++	+++
Parabrachial nucleus	+++	++

In the midbrain they found the largest numbers of LepRb-positive neurons and projections in the dorsal raphe and periaqueductal gray. The hindbrain had LepRb positive neurons in and within surrounding areas of the nucleus of the solitary tract (NTS). NTS also contained a high number LepRb projections but the LepRb projections in the area surrounding NTS were considerably stronger. The authors found no expression of LepRb in the area postrema (AP) or LepRb positive projection, suggesting that leptin is not signalling in this area [205].

These results are fairly consistent with a transgenic mice used by Scott and colleagues [206] where the leptin receptor promoter is driving *Cre* and containing a *Cre*-inducible LacZ. Scott and colleagues also investigated LepRb neurons ability to sense peripherally administered leptin by looking a phosphorylated STAT3 (pSTAT3), an indicator of active signalling by the leptin receptor. Phosphorylation of STAT3 has previously been used as described by Ghilardi and colleagues [207]. In Scott *et al.,* paper they found few of the brain areas expressing LepRb to have STAT3 phosphorylation after administration of leptin in the periphery. These areas included region of NTS, hypothalamus and medial preoptic nuclei (MPO). Areas that had STAT3 phosphorylation were in general adjacent to cerebral ventricles or aqueduct.

Garfield and colleagues further characterized the two different transgenic animals by validate their expression with leptin-induced pSTAT3 staining in the NTS [208]. They revealed a high consistency with these two different transgenic animals and demonstrated a 70% and 80% co-localization of the reporter strain and pSTAT in the NTS.

1.5.5.2 Leptin receptor expression in pancreas

Several research groups have confirmed the presence of the leptin receptor in pancreatic cells as listed in table 1.5. Kieffer and colleagues [173], were the first to demonstrate that the leptin receptor was expressed in pancreatic β-cells by Northern Blot, RT-PCR and Southern blot. However, they only confirmed the presence of the extracellular domain that does not include the vital signalling part of the leptin receptor [143].

Table 1.5 Detection of LepR in islet		
Method	**Model**	**Ref.**
Northern blot, RT-PCR, southern blot	Rat islets and insulinoma cell line (β TC-3) *extracellular domain	[173]
RT-PCR	Rat islets (extracellular weak detection and intracellular domain very weak)	[185]
Southern blot on RT-PCR and northern blot	WT and *Ob/Ob* mice islets and pancreas *intracellular domain detection	[174]
Southern blot on RT-PCR product	Rat islets, insulinoma cell lines and mouse pancreas *intracellular domain detection	[179]
RT-PCR, Cy3-leptin	Rat islets, insulinoma cell line (INS-1), δ-cells (RINB2), and α-cells (INR1G-9) *intracellular domain	[175]
RT-PCR	Rat islets *intracellular domain	[180]
RT-PCR, Cy3-leptin	Primary human islets *intracellular domain	[178]
RT-PCR	*ob/ob* mice islets *intracellular domain	[209]
RT-PCR	INS-1, hamster (HIT), and mouse (β-TC-3), and INR1-G9	[190]
RT-PCR	Rat islets, MIN-6, RIN6	[182]
RT-PCR	Rat islets *intracellular domain	[176]
Reporter strain	Mice islets	[210]
Single cell RNA-seq	Human islet cells	[211]
Single cell RNA-seq	Human islet cells	[212]

Leqlerc-Meyer and colleagues [185] demonstrated that most of the leptin receptor expressed on the β-cells lacks the vital signalling domain. In contrast, another group demonstrated leptin receptor expression of the intracellular domain using RT-PCR in mouse islets and pancreas and with northern blot in *ob/ob* mice islets [174] in addition, they detected expression in kidney, liver, heart and spleen but not in white adipose tissue. Several groups have with RT-PCR confirmed the leptin receptor expression in isolated rat islets [175, 176, 180, 182] and in adipose tissue in mice [182].

The Kieffer research group also looked at the distribution in different islet cells demonstrating that leptin binds to insulin and somatostatin producing cells but not to glucagon cells, consistent with data from cell lines [175]. In contrast, one research study have demonstrated an expression of the leptin receptor in a α-cell

line (α-TC1-9) and mouse and human α-cells [99]. In a second publication, the Kieffer group demonstrated with RT-PCR and Cy3-leptin the expression of leptin receptors in human islets [178].

In contrast to these results demonstrating an expression of the LepRb in islets, Fujikawa and colleagues [210] used a leptin receptor promoter driving *Cre* crossed with a *Cre*-reporter mice strain and found no expression in islets as shown in figure 1.6. In FACS sorted human islets no expression with RNAseq of LepRb was detected in α-, β- or γ-cells but the δ-cells had an enriched expression of LepRb [211, 212].

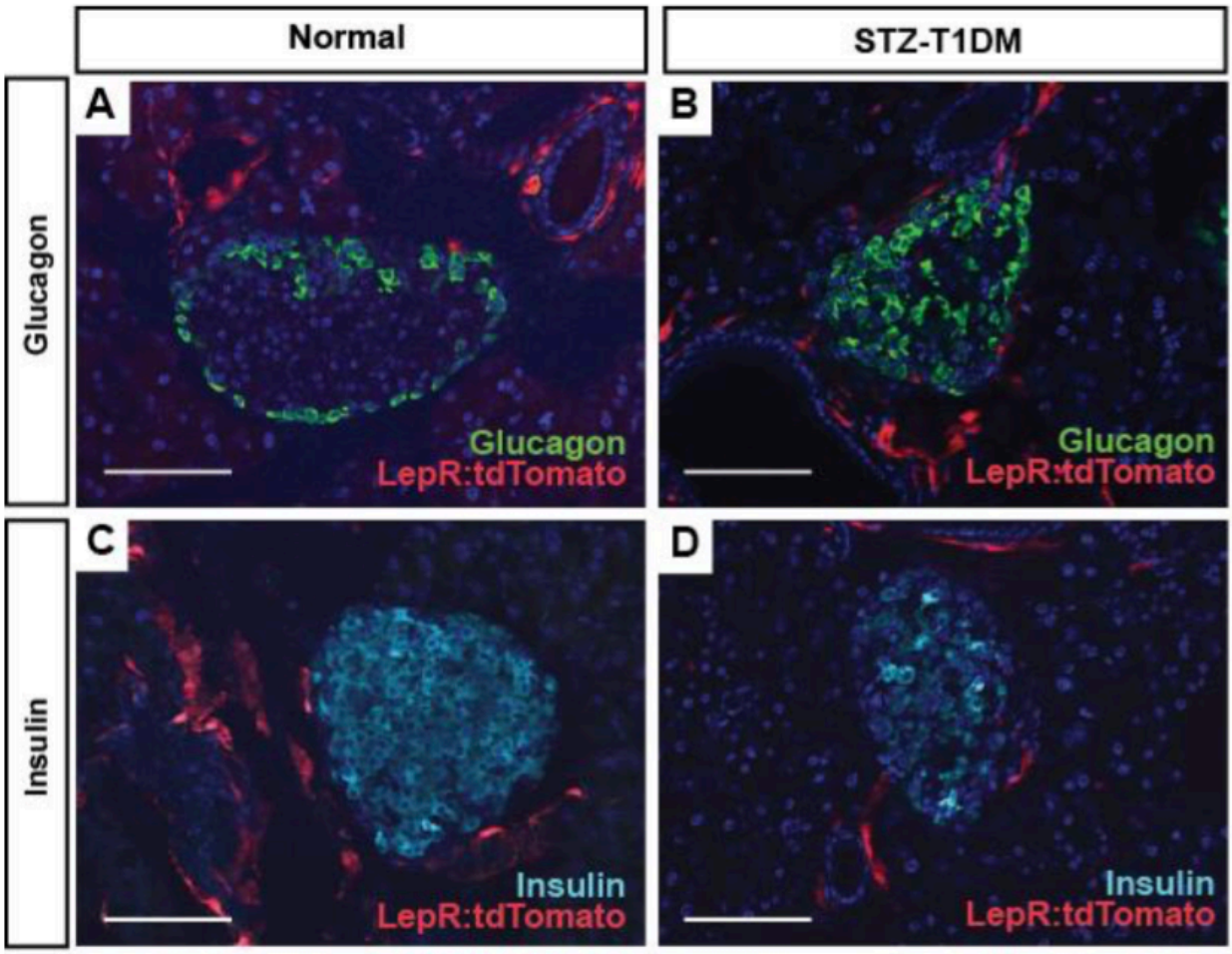


Figure 1.6 - LepRb is not expressed by pancreatic insulin- or glucagon-producing cells (A) Distribution of cells expressing LepRb (red) and glucagon (green) in pancreas of normal **(A)** and STZ-induced insulin deficient **(B)** LepR-ires-Cre;tdTomato$^{flox/flox.}$. **(C)** Distribution of cells expressing insulin (sky-blue) and LepRb (red) in same animals as **(A)** and **(B)**. Image is taken from [210]

It is worth to mention that the majority of research studies have used RT-PCR to identify the presence of leptin receptors on pancreatic cells. None of the research reports have demonstrated a quantitative measurement of LepRb expression from islets.

1.6 Central regulation of energy homeostasis and glucose homeostasis

In the present research study we are interested in how the brain senses peripheral metabolic signals and in particular how the brain regulates pancreatic hormone secretion but also how it regulates food intake. Several brain circuits in hypothalamus are involved in sensing peripheral signals and responding to them affecting food intake or glucose regulation. We are also interested in a subset of neurons in the hypothalamus and GLP-1-neurons of the NTS and the possibility that they are involved in central regulation of energy homeostasis and/or glucose homeostasis.

Peripheral factors send information to the brain to regulate food intake and energy balance. For instance, leptin secreted from the adipose tissue informs the brain about fat reserve and the brain responds by increasing satiety. The brain receives these signals via two different pathways the neural pathway or the humoral pathway. The humoral pathways consist of the circumventricular organs CVO (not fully insulated from the BBB), where the brain receive information from the periphery of energy balance in addition to hormones that can directly enter the brain.

Signals from the periphery influencing the central regulation of energy homeostasis are mainly of two categories. One is the satiety signals generated after a meal causing the feeling of fullness and satiety that stops you eating and prolongs the time until hunger reappears.

There are two types of satiety signals: short-term and long-term satiety signals. Short-term satiety signals involve gastric distension and the release of peptides from the gastrointestinal tract. A variety of endogenous peptides have been identified and found to affect feeding [213]. After feeding the gastric distension is sensed by sensor receptors in the stomach and information is sent via the vagal afferent and spinal sensory nerves to the hindbrain. Most of the peptides also exert their effect via vagal afferent fibres but some also enters the brain via the humoral pathway [214]. The afferent signals are processed initially in the hindbrain with nucleus of the solitary tract (NTS) playing an important role in processing the vagal afferent signals but the area also receives input directly from circulating peptides via the adjacent area postrema.

The peptides involved in short term satiety includes cholecystokinin (CCK) [215], glucagon like peptide-1 (GLP-1), oxyntomodulin, peptide (PYY), apolipoprotein A-IV, and enterostatin. In addition, several peptides are secreted from pancreas such as pancreatic polypeptide, glucagon and amylin. Ghrelin is a hormone released from the gastric mucosa in contrast to satiety peptides stimulates feeding [216-218].

On the long-term satiety control of feeding and energy homeostasis is the hormone leptin. Leptin acts on the brain to regulate adiposity by constraining fat mass and decreasing food intake. However, leptin can also in

response to short term changes in energy balance change in plasma levels [219]. The different peptides are discussed in more detail in section 1.6.3.

1.6.1 Hypothalamic regulation of food intake

To balance food intake, energy expenditure and body fat reserves the hypothalamus receives afferent signals from the periphery and sends back efferent messages. The main areas of hypothalamus involved in the regulation of food intake are ventromedial (VMH), paraventricular nuclei (PVN), lateral hypothalamus (LHA) and the arcuate nucleus (ARC), Figure 1.7.

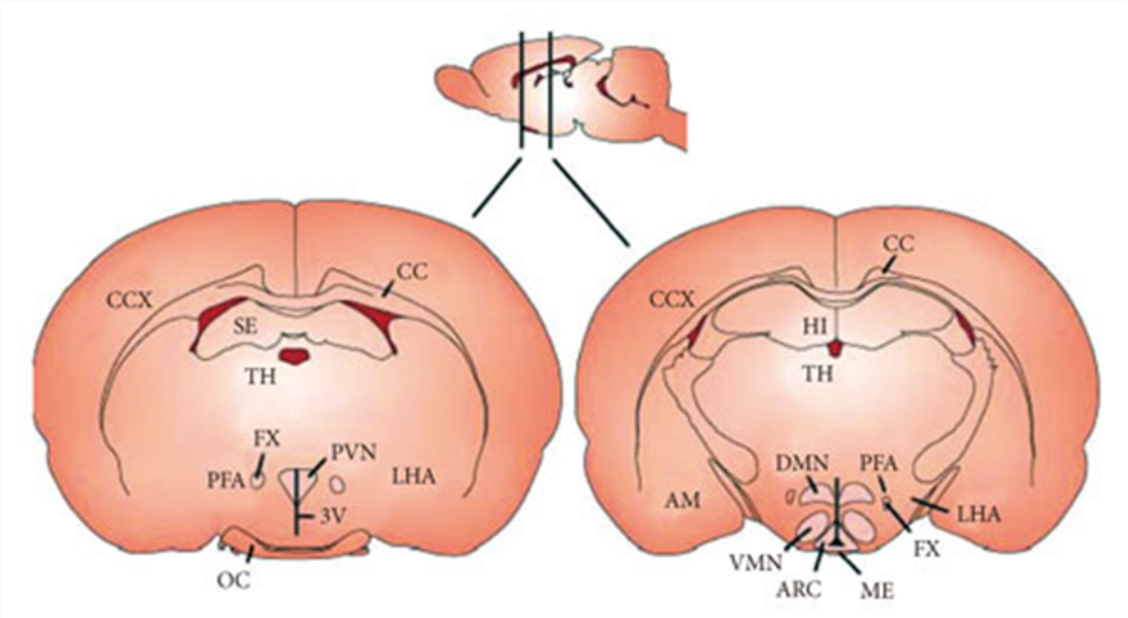


Figure 1.7 - Main hypothalamic areas involved in appetite regulation. Hypothalamus is located centrally below thalamus (TH) and surrounding the third ventricle (3V). **Left side**: Paraventricular nucleus (PVN), lateral hypothalamic area (LHA), cerebral cortex (CCX), corpus callosum (CC), septum (SE), fornix (FX), optic chiasm (OC) and the perifornical area (PFA).**Right side**: The arcuate nucleus (ARC) is located adjacent to the median eminence (ME). Ventromedial nucleus (VMN), dorsomedial nucleus (DMN), lateral hypothalamic area (LHA). Also indicated is: amygdala (AM) and the hippocampus (HI). The figure is from [220].

Hormones enters hypothalamus from the circulation via the blood brain barrier and when inside the brain interacts with their receptors expressed on specific neurons [221].

1.6.1.1 Arcuate nucleus of hypothalamus

One of the most important areas in the regulation of energy balance is the arcuate nucleus (ARC) of hypothalamus. The ARC plays a key role in the regulation of appetite and body weight. The nucleus is located next to median eminence (ME), one of the brain's several circumventricular organs and it surrounds the lower part of the third cerebroventricle (3V). Thus, hormones, nutrients and cerebrospinal fluid can easily access ARC and the area is considered to be the primary sensor of metabolic signals from the periphery

[222]. The ME is connected to the ARC by tancytes that are specialized hypothalamic glia cells. The tancytes forms a barrier between the blood circulation and CSF [223] and for instance are responsible for the entry of leptin into hypothalamus [224].

The ARC contains two main populations of neuroendocrine neurons involved in growth and lactogenesis. The area also contains centrally projecting neurons with different roles in the regulation of feeding behaviour. These neurons include the pro-opiomelanocortin (POMC) neurons expressing the appetite suppressing neuropeptide proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART). A second type of neurons is appetite stimulators neuropeptide Y (NPY) and agouti-related protein (AgRP) neurons [225, 226]. These neurons are first order neurons responding to afferent signals from adipose tissue such as leptin, gastrointestinal tract (ghrelin), and nutrients [227], these two neuronal populations then communicate with other areas in hypothalamus such as PVN, DMN and LHA [228]. There is also a third neuronal population in the ARC that is less defined, named RIP2*Cre* neurons [229].

1.6.1.1.1 POMC/CART anorexigenic neurons of the ARC

POMC-neurons are intermingled with the NPY/AgRP neurons and are either excitatory or inhibitory releasing the neurotransmitter glutamate or GABA [230].The POMC-neurons also produce α, β, γ-melanocortins and the adrenocorticotropic hormone (ACTH) via post-translational processing from the POMC-peptide. Deletion of all POMC produced peptides results in an increase in food intake and weight gain [231]. The melanocortins binds to the melanocortin receptors (MC-Rs) with two different types expressed on secondary neurons in the brain (MC3-R and MC4-R), mostly in the PVN [232]. Ablation of the MC4-receptor in mice results in obese animals with a reduced energy expenditure [233].

Most of the POMC-neurons also co-express CART mRNA. Several animal studies have shown that administration of CART inhibits food intake, and the injection of CART antiserum increases the food intake [234]. However, animals deficient of CART display no significant change in food intake nor in body weight [235]. Other studies have also demonstrated an orexigenic role of CART. Thus, injection of CART into the VMN or ARC in fasted rats led to an increase in food intake [236].

POMC-neurons in the ARC reduce the food intake by releasing one of the melanocortins, the α-MSH (melanocyte-stimulating hormone) that activates the melanocortin receptor [237]. The NPY/AgRP neurons have counteracting effects by inhibiting the POMC-neurons by the release of AgRP (an inverse agonist to the melanocortin receptors) [238]. POMC-neurons also respond to the monoamine serotonin via a 5HT-2C receptor to inhibit food intake and promote weight loss [239].

1.6.1.1.2 NPY/AgRP orexigenic neurons of the ARC

NPY neurons in hypothalamus are important regulators of food intake and energy expenditure and thereby body weight. NPY acts on five different receptors (Y1-Y5), the main one where NPY exerts their orexigenic effect are Y1 and Y5 receptors [240, 241]. Neurons expressing NPY are mostly in the arcuate nucleus and the majority co-express AgRP [242]. Deletion of the AgRP neurons in adult mice leads to rapid starvation whereas deletion during the neonatal stage had minimal effect on feeding [243-245] central injection of NPY increases food intake [246].

The neurons are activated during energy deficit by the circulating peptide such as ghrelin [247], or during energy surplus by leptin [248] and insulin [249]. NPY neurons have high number of projections into the other hypothalamic nucleus such as PVN, VMN, DMN and LHA and this is the main areas the NPY neurons exerts its orexigenic effect (Figure 1.8). Around one fifth of the NPY/AgRP neurons projects into DMN and PVN [250].

1.6.1.1.3 RIP2*Cre* neurons of the ARC

The arcuate nucleus also contains a third neuronal population intermingled with the POMC/CART and NPY/AgRP-neurons that are suggested to have a role in energy homeostasis. These neurons do not express POMC or NPY peptides and are thereby distinct from these neurons [229]. RIP2*Cre* neurons were found whilst using transgenic mice harbouring the rat insulin 2 promoter driving *Cre* (RIP2*Cre*) transgene [251]. The RIP2*Cre* neurons are discussed in more detail in section 1.7.

1.6.1.2 Paraventricular nuclei (PVN) of hypothalamus

The PVN is a very important downstream second order structure to the ARC neurons. POMC/CART, NPY/AgRP and RIP2*Cre* neurons located in the ARC all share targets in the CNS, one of them being the PVN [250, 252]. Injection into the PVN of any of the orexigenic signalling peptides such as NPY, orexins and GABA stimulates feeding. The NPY/AgRP-neurons innervate neurons in the PVN that express thyrotropin releasing hormone (TRH) [253] and this hormone has been demonstrated to exert an effect on food intake and energy expenditure [254].

The PVN also contain neurons producing the corticotropin-releasing factor (CRF) [255]. Central administration of CRF into rats or mice induces anorexia [256, 257], it has also been found to lead to hypermetabolism and an increased brown fat thermogenesis [258-260].

As mentioned in section 1.6.1.1, neurons in the PVN express MC3-R and MC4-R that responds to melanocortins produced by the POMC-neurons as well as the antagonist AgRP. It has been suggested that around 6% of early onset obesity in humans is due to mutations in the MC4-R including ninety different mutations [261].

1.6.1.3 Lateral hypothalamic area (LHA)

The lateral hypothalamus (LHA) contains two types of neuronal populations; one is the orexin-(hypocretin), expressing neuronal population and the other one is the melanin- concentrating hormone (MCH) expressing neurons. Orexin is a peptide that is involved in feeding behaviour as well as arousal. The other peptide involved in stimulating food intake. The area receives several neuronal projections from the ARC. NPY/AgRP neurons and α-MSH positive neurons are concentrated here and are in contact with the orexin and MCH-expressing neurons [262].

MCH-expressing neurons from LHA further project into the brainstem, spinal cord and cortex [263]. Deletion of the MCH receptor (Mchr) in mice leads to animals with an elevated energy expenditure, increased locomotor activity and resistance to diet induced obesity [264]. Administration of the orexin peptides A or B or an antagonist reduces food intake [265, 266]. Both NPY and POMC- neurons in the ARC innervate the orexin-expressing neurons [267].

1.6.1.4 Dorsomedial nucleus (DMN)

Removal of the dorsomedial nucleus of hypothalamus leads to animals suffering from obesity due to hyperphagia [268]. Several of the NPY-neurons originating from the ARC projects into this area as well as α-MSH positive neurons [269]. Several of α-MSH positive neurons in the DMN also projects to the PVN to innervate TRH-positive neurons [270].

1.6.1.5 Ventromedial nucleus (VMN)

Electrolytic or chemical lesions have demonstrated that this area is involved in inhibition of food intake and increases metabolism [271]. The ventromedial nucleus is an area harbouring a high percentage of the glucose responsive neurons (glucose responsive cells are discussed in section 1.6.4.1). Studies in humans with neuroimaging have revealed that after oral glucose ingestion VMN is activated [272].

The brain derived neurotrophic factor (BDNF) is highly abundant amongst the neurons in the VMN and the factor has been demonstrated to decrease food intake and body weight after central administration [273]. Neurons in the VMN also express LepRb and this area is partly mediating leptin's effect on energy

homeostasis alongside the ARC and other areas [274]. It is suggested that POMC-neurons originating from the ARC have a role in activating BDNF neurons to inhibit food intake [275].

Several neurons within VMN express the nuclear receptor, steroidogenic factor 1 (SF1). The SF1-neurons regulate energy balance and glucose homeostasis [276, 277]. Both LepRb and InsR are expressed on the neurons and selective deficiency of these receptors in the neurons results in the opposite body weight phenotype [166, 278].

Table 1.5 Centrally released signalling peptides

Orexigenic (stimulate food intake)	**Anorexigenic (inhibits food intake)**
Neuropeptide Y (NPY)	Cocaine and amphetamine-regulated transcript (CART)
Agouti gene related protein (AgRP)	α-melanocytes-stimulating hormone (α-MSH)
GABA	Corticotropin-releasing factor (CRF)
Orexin A and B	brain derived neurotrophic factor (BDNF)
Melanin-concentrating hormone (MCH)	thyrotropin releasing hormone (TRH)
GHRH	Serotonin

1.6.2 The brainstem and the dorsal vagal complex

Like the median eminence, the area postrema (AP) located in brainstem contains an incomplete blood brain barrier (circumventricular organ) and therefore via this structure the brainstem can receive peripheral appetite signals. Several neuronal pathways between the brainstem and the hypothalamic circuits and other brain centres exist [279, 280]. In addition, the vagus nerve links the gastrointestinal area with the brain with the cell bodies of the afferent fibres located in the nodose ganglia and the axons terminating in the dorsal vagal complex (DVC) in the brainstem. The DVC consists of the nucleus of the solitary tract (NST), the AP and the dorsal motor nucleus of the vagus (DMNV) [281].

In this study, we are interested in the leptin receptor signalling in neurons of the NTS in particular GLP-1 neurons and their possible effect on feeding behaviour and glucose regulation. The GLP-1 neurons are located in an area receiving peripheral input including leptin.

1.6.2.1 GLP-1 expressing neurons of NTS

In addition to being an incretin, GLP-1 is expressed in the brain by GLP-1 neurons also referred to as pre-proglucagon (PPG) neurons. These neurons are primarily located in the lower part of the brainstem, concentrated within the nucleus of the solitary tract (NTS) [134, 282-284].

At present, the role of peripheral GLP-1 and the central GLP-1-expressing neurons and their interplay is not fully understood, nor are their interactions with therapeutic GLP-1 analogues [285]. It is contested whether peripheral secreted GLP-1 actually can reach its receptor (GLP-1R) located in the brain because the majority of GLP-1R are located inside of the BBB and it is not clear if peripheral GLP-1 can cross this barrier [285]. Secondly, considering that peripherally-secreted GLP-1 has a very short half-life in the circulation it is more likely that GLP-1 within the brain is activating GLP-1R in the brain [285].

The development has recently been described of a transgenic mouse expressing YFP under the control of the glucagon promoter [286]. These mice have strong YFP expression in GLP-1 neurons allowing visualisation of not only in the cell bodies and terminals but also axons. Use of this transgenic mouse revealed that the GLP-1 neurons in the NTS project extensively to central autonomic regions involved in metabolic and autonomic control, including the ARC, PVN, DMN and the brainstem nuclei [287, 288].

In another study, the above group investigated the location of the GLP-1R location in the brain with help of a transgenic mouse where the GLP-1 receptor promoter is driving *Cre* as well as harbouring a *Cre* reporter for detection [123]. Several GLP-1R were found to be expressed close to the circumventricular organs, amygdala, several hypothalamic nuclei and a few receptors were found to be located in the NTS.

Several studies have shown that injection of GLP-1 or GLP-1 agonists into the brain leads to the suppression of food intake [289, 290]. Interestingly, in one research study the authors found that an obstruction of GLP-1R in the brain led to animals with an impaired glucose tolerance suggesting a role for central GLP-1 in glucose homeostasis regulation [291]. In the latter study they injected a GLP-1 receptor antagonist into the ARC of rats and observed impaired glucose tolerance. Several GLP-1 positive fibres originating from the NTS terminate in the ARC, suggesting that GLP-1R effects on glucose homeostasis could be under the control of GLP-1-expressing neurons in the NTS. A central role for GLP-1 in the regulation of glucose homeostasis is further supported by a group that also blocked the GLP-1R in the CNS and found animals to be hyperglycemic and glucose intolerant [292]. In addition, they observed no effect on body weight but an increase in food intake. In other studies they have found an effect of centrally administered GLP-1 to increase blood pressure and heart rate [293, 294].

1.6.2.2 Leptin's effect on GLP-1-expressing neurons

Several studies have demonstrated that leptin has an effect on brainstem neurons, including the GLP-1 neurons. Peripheral administration of leptin has been demonstrated to activate C-Fos expression, a marker for neuronal activity, in GLP-1 neurons in the NTS of mice [295]. Secondly, the peripheral administration also increased pre-proglucagon expression in the NTS of mice [296]. However, it has also been

demonstrated that leptin injected on its own does not increase C-Fos activation whereas in combination with CCK or gastric preload administration it increase the C-Fos activation [297].

ICV administration of leptin into the fourth ventricle in rats has been demonstrated to have an inhibitory effect on food intake and body weight after administration of leptin [298]. This is consistent with injection of leptin into the caudal brainstem which effectively suppressed food intake when applied in combination with mechanical distension of the stomach [299].

The leptin receptor signalling in the NTS and AP has been perturbed via AAV-shRNAi mediated knock-down in rats, leading to animals that were hyperphagic and with increased adiposity [300].

Hisadome and colleagues have shown that leptin directly depolarizes GLP-1 neurons in the NTS suggesting that these neurons are responsive to leptin [301]. In an *in vivo* study by Scott and colleagues, LepRb was deleted using a *Cre*-recombinase expressed under the paired-like homeobox 2b (Phox2b) promoter, expressed exclusively in the autonomic nervous system and not in hypothalamus [302]. LepRb was found to be ablated in neuronal populations in the brainstem and these neuronal populations were proposed to include GLP-1expressing neurons of the NTS. Animals were found to have a normal glucose tolerance but were hyperphagic and had a 10% increase in bodyweight. However, Scott and colleagues targeted several neuronal populations, most likely including catecholaminergic neurons as well as efferent vagal neurons. These research studies suggest that LepRb signalling in NTS are required for normal energy balance control and that GLP-1 expressing neurons could mediate part of this effect.

Garfield and colleagues have further characterized the neurons expressing LepRb in the NTS by investigating their neurochemical profile. They demonstrated that several neuronal populations involved in in energy regulation such as ChAT, nNOS, nesfatin, PrRP or CART do not express the leptin receptor. However, 62.6% of the leptin receptor expressing neurons in NTS expressed GLP-1 mRNA and 43% expressed CCK mRNA. They further demonstrated that there is no overlap with POMC neurons suggesting the GLP-1 are a discrete subpopulation in the NTS [208].

1.6.3 Peripheral hormones affecting energy homeostasis

The ingestion of food results in the activation of vagal afferents that turn projects to hypothalamus as well as the brainstem to process the signals. In addition, peripheral hormones secreted from the pancreas, adipose tissue, the stomach and several gut hormones are secreted in response to feeding mainly from L-cells in the gut. These hormones are released into the circulation and act on the hypothalamus and the brainstem. Several of these gut hormones are secreted from the L-cells of the gut. In this study we delete LepRb in cells expressing preproglucagon which includes the L-cells, pancreatic α-cells and the GLP-1 neurons. The GLP-1

in the NTS located in the area responding to the peripheral hormones and therefore might be responding or involved in this network.

The gastrointestinal system releases several different hormones secreted in response to feeding. The hormones circulate and reach the brain via circumventricular organ and activate the different neuronal pathways involved in regulation of food and energy balance (Figure 1.8).

Orexigenic peptides

1.6.3.1 Ghrelin

Ghrelin has the opposite effect to leptin, and increases hunger. Ghrelin is mainly produced in the periphery and acts on the growth hormone secretagogue receptor (GHS). The hormone is produced by X/A cells that constituents of around 20% of the endocrine cell population in the oxyntic glands in humans [303]. There are also neurons in hypothalamus producing ghrelin [218, 304], as well as the ε cells of pancreatic islets [9].

Injection of ghrelin into the PVN of rats initiates hunger and increases food intake [217]. It has been observed that blood ghrelin levels are decreased in human obesity [305]. A plasma concentration of ghrelin increases during fasting and decreases post-prandially. However, total ablation of the hormone in mice had no effect on neither appetite nor body weight [306].

Anorexigenic peptides

1.6.3.2 Peptide tyrosine tyrosine (PYY)

Peptide tyrosine tyrosine (PYY) is produced and released into the bloodstream by intestinal L-cells after food intake. The peptide belong to the PP-fold family comprising of NPY, PYY and PP. Administration of PYY reduces food intake [307] and mice deleted for the peptide suffer from obesity [308]. Meals rich in proteins cause the largest increase of PYY concentrations in the bloodstream [309].

1.6.3.3 Pancreatic polypeptide (PP)

The pancreatic polypeptide (PP) belonging to the PP-fold family of peptides is produced and secreted into the circulation from pancreatic cells after a meal and is like PYY secreted in proportion to the amount of calories ingested [310]. Peripheral administration of PP leads to a reduction in food intake in both rodent and humans [311, 312] and activates neurons in the area postrema [313]. Food intake and fat mass is reduced in

mice overexpressing PP [314]. Chronic administration of PP into obese mice has been demonstrated to reduce the weight gain and food intake [313].

1.6.3.4 Cholecystokinin (CCK)

Cholecystokinin (CCK) has been found to inhibit food intake in rodents [215] and humans [315]. The hormone is released after meal intake from the intestine and it has been demonstrated to co-localize with PYY in L-cells [316]. An antagonist against the CCK receptor has been demonstrated to increase food intake and rats that lack the CCK receptor are hyperphagic and obese [317, 318].

The effect on food intake is suggested to be mediated by receptors on the vagus nerve that relays to the brainstem as shown in figure 1.8. However, it has also been demonstrated that central administration of CCK into DMN and PVN of hypothalamus decreases food intake [319].

1.6.3.5 Glucagon like peptide 1 (GLP-1)

It is suggested that central administration of GLP-1 regulates energy balance and peripheral GLP-1 regulates glucose homeostasis. However, there is evidence that peripheral GLP-1 also is involved in regulation of energy balance. As previously mentioned it is unclear whether peripheral secreted GLP-1 can reach the receptors expressed in the brain and exert its inhibition of food intake as reviewed in [284, 285, 320]. Instead, it is suggested that peripheral GLP-1 acts via the vagus nerve to inhibit food intake [321].

1.6.3.6 Oxyntomodulin

Oxyntomodulin is a product of the pre-proglucagon molecule (Figure 1.3) and is converted into oxyntomodulin in L-cells. Oxyntomodulin reduces food intake when administered peripherally to rodents or humans and centrally into rodents [322-325]. It has been demonstrated that oxyntomodulin binds to the GLP-1 receptor, albeit with lower affinity. Oxyntomodulin's effect on food intake is blocked by the GLP-1 receptor antagonist exendin 9-39 [322] and is missing in GLP-1 receptor knockout animals [326]. The two hormones have a similar neuronal activity pattern after peripheral administration [326].

1.6.3.7 Amylin or amyloid polypeptide (IAPP)

Amylin is co-localized and co-released with insulin from the β-cells. The peptide is proposed to have an effect on food intake by acting on receptors in the brain and via a peripheral mechanisms and indirectly by acting on gastric emptying [327].

1.6.3.8 Leptin

Leptin is a satiety hormone secreted from the adipose tissue and is a strong suppressor of food intake (the hormone is discussed in more detail in Section 1.6).

1.6.3.9 Insulin

Insulin, in addition to its action on peripheral tissues regulating glucose homeostasis, the hormone has receptors located in the brain. The activation of centrally located receptors has an effect on peripheral metabolism such as appetite regulation, body temperature, hepatic glucose output and responding to hypoglycaemia [328-332]. Central injection of insulin induces anorexia by decreasing NPY and stimulation of POMC expression [333].

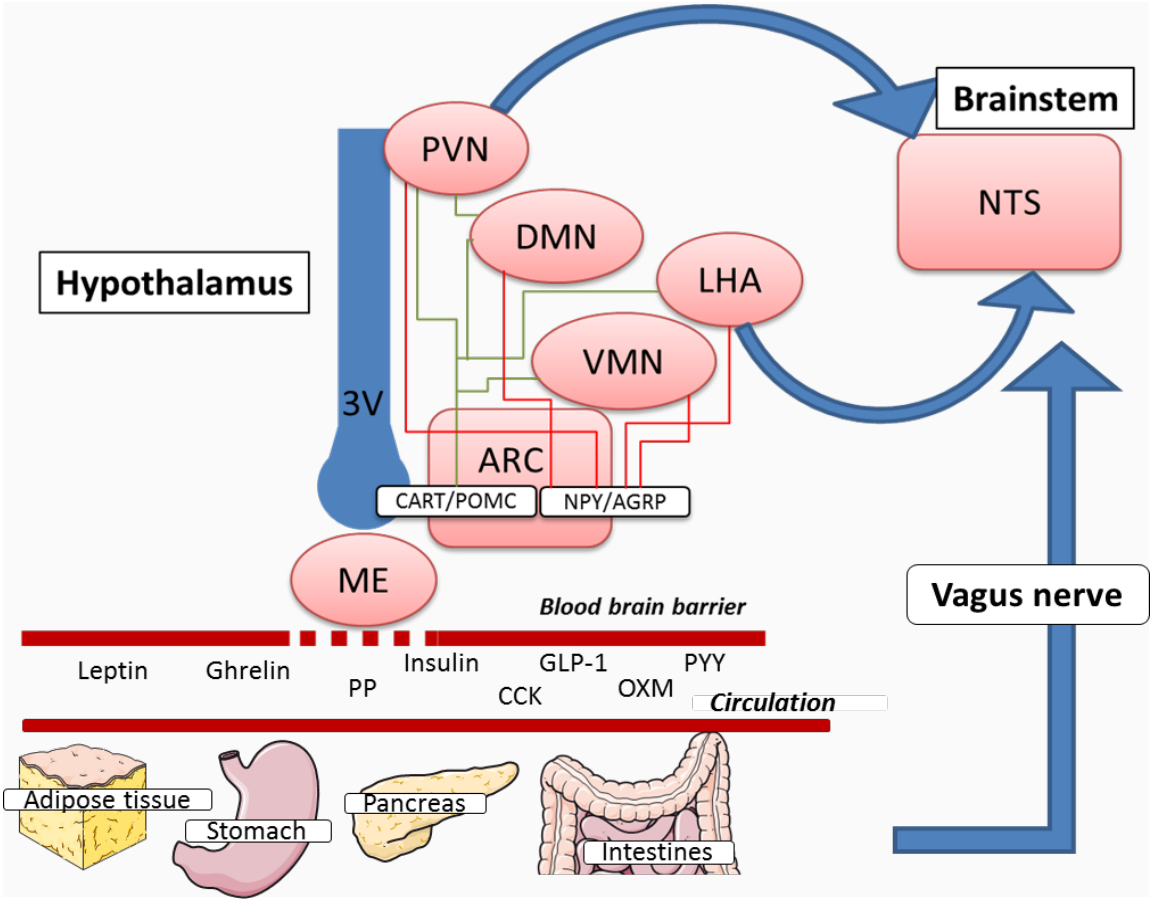


Figure 1.8 - Hypothalamic areas and peripheral hormones involved in appetite regulation. Peripheral hormones in the circulation derived from the gut: CCK, PYY, OXM, GLP-1, the stomach (ghrelin), pancreas derived insulin and pancreatic polypeptide and the adipose derived leptin reaches the brain via the leaky blood brain. Inside the brain the hormones either stimulate or inhibits the CART/POMC-neurons and NPY/AgRP-neurons. Projections from the ARC reach the other hypothalamic areas and the brainstem. Several peripheral signals reach the NTS via the vagus nerve. CCK=Cholecystokinin, PPY=Peptide tyrosine tyrosine, OXM=Oxyntomodulin, GLP-1=Glucagon like peptide 1, PVN=paraventricular nucleus, DMN= dorsomedial nucleus, LHA=lateral hypothalamic area, VMN=ventromedial nucleus, ARC=arcuate nucleus, ME=median eminence and 3V=third ventricle, NTS=nucleus of the solitary tract. The figure is generated from images from servier medical art.

1.6.4 Central nervous system role in controlling glucose homeostasis

The endocrine pancreas is not unique in being able to detect glucose and respond to fluctuating glucose levels: in addition, several organs located throughout the body such as the portal vein, gut and the brain are also involved. The brain contains several neuro-circuits that detect and integrate signals of glucose levels and control peripheral tissue to respond to changes in glucose levels. The system is referred to as the brain centred gluco-regulatory system (BCGS) and acts via insulin dependent and insulin independent pathways [334]. Considering that the brain has a high glucose demand it has even been proposed that the brain is the main sensor of glucose and that hypothalamus is the main centre for glucose sensing [335].

1.6.4.1 Central glucose sensing

The role of the brain in controlling glucose homeostasis was first demonstrated by the physiologist Claude Bernard in 1854. Dr Bernard observed that diabetes (glycosuria) could be induced after puncturing the floor of the fourth ventricle located in the lower brainstem of a rabbit brain [336]. The importance of this discovery was neglected after the discovery of insulin by Banting in 1923. However, after the discovery of glucose sensing neurons in hypothalamus [337, 338] central regulation of glucose homeostasis has been further investigated.

The brain can sense glucose in the extracellular fluid in the brain via their glucose responsive neurons (glucose-excited and glucose-inhibited neurons). These neurons are widely distributed in the brain but concentrated in hypothalamus and the brainstem and have a role in maintaining normal glucose levels [339]. Glucose-excited neurons are mainly located in brain areas such as ventromedial hypothalamus (VMH), the arcuate nucleus (ARC) and the paraventricular nuclei (PVN). On the other hand, the glucose-inhibited neurons are located mainly in the lateral hypothalamus LH, ARC and PVN [340]. Both of the two different types of neurons are also located in several areas of the brainstem in the dorsal vagal complex that includes NTS, area postrema and the dorsal motor nucleus of the vagus (DVM)[341].

Glucose-sensing cells are also located in the periphery including in the hepatic portal vein. These cells sense the circulating glucose and form a network monitoring blood glucose at these different locations and either activate or inhibit the sympathetic and parasympathetic nervous system [85].

1.6.4.2 Central insulin sensing

In addition to glucose sensing neurons, the brain also have the insulin receptor (IR) widely distributed in several areas of the brain including areas such as hypothalamus [342]. It has been demonstrated that antagonism of hypothalamic insulin signalling leads to weight gain, hyperphagia, insulin resistance and

decreased hepatic glucose production (HGP) [332]. Destruction of several different brain regions has been demonstrated to affect pancreatic hormone secretion. Lesion of VMN results in hyper secretion of insulin and an increase of glucagon levels [343].

1.6.4.3 Central control of pancreatic hormone secretion

For the brain to be able to exert an effect on pancreatic islet hormone secretion it either has to have a direct effect by innervation of the islets or by modifying circulating factors that influence islets hormone secretion. In fact, pancreatic islets (at least in rodents) are highly innervated by parasympathetic (vagus) and sympathetic (splanchnic) nerves [86]. Furthermore, both α and β-cells express neurotransmitter receptors on their surface. The α-cell expresses β2-adrenergic receptors and β-cells express the muscarinic acetylcholine receptor (m3AchR) and α2-adrenergic receptors [344].

During feeding, glucose reaches glucose-sensing cells in the hepatic portal vein area that activate neurons in the brainstem and hypothalamus via the vagus afferent nerve. In addition, glucose is being sensed directly by the specialised neurons located in hypothalamus and the brainstem. In both situations the brain exerts its control of insulin secretion via parasympathetic nervous system. Upon activation, the parasympathetic nerve endings releases acetylcholine that binds to m3AchR expressed on β-cells to increase insulin secretion [345].

Upon hypoglycemia the sympathetic nervous systems stimulate glucagon secretion. Norepinephrine is released from the sympathetic terminals activating the β2-adrenergic receptor on the α-cells to release glucagon [346]. At the same time the norepinephrine release inhibits insulin secretion by activating α2-adrenergic present on β-cells [347].

It should be emphasised that the extent and importance of innervation of islet cells in the human pancreas is less well-established.

1.7 Liver kinase B1 (LKB1) and its role in RIP2*Cre* neurons of the ARC

In our last research project we are interested in the tumour liver kinase B1 (LKB1) and its possible roles in the brain of adult mice. Considering LKB1 is an upstream target of the nutrient sensing kinase AMP-activated protein kinase (AMPK) with known roles in energy homeostasis there is a possibility that LKB1 has similar roles.

1.7.1 Liver kinase B1 (LKB1)

LKB1 is a serine/threonine kinase that directly phosphorylates adenosine monophosphate-activated kinase (AMPK) and 12 other AMPK-like kinases and thereby regulates several important cellular functions such as metabolism, growth, autophagy and polarity [348, 349]. It is a tumour suppressor and mutation inactivating the suppressor is found in the majority of patients with Peutz-Jeghers syndrome. These patients develop benign and malignant tumours of several organs [350]. A systemic deletion of LKB1 is lethal and mice die at the embryonic stage due to defects in the vasculogenesis homolog and placental development [351]. The ubiquitously expressed LKB1 is transcribed into a large 433 amino acid [352]. For LKB1to be functional it needs to form a complex with Ste20-related adaptor (STRAD) and mouse protein 25 (MO25) [353].

LKB1 is suggested to play a role as an upstream regulator of the phosphatase and tensin homologue (PTEN) via phosphorylation and protein interaction and/or upregulation of PTEN expression [354]. PTEN is a negative regulator of PI3K/Akt pathway and is mutated in several types of cancers [355].

1.7.2 LKB1 an upstream regulator of AMP-activated protein kinase (AMPK)

AMPK is a serine/threonine protein kinase which functions as a sensor of cellular energy status. During a cellular energy decline AMPK is activated due to the increase of AMP:ATP or ADP:ATP ratio. AMPK acts to restore the cellular energy by activating processes that generates ATP and by inhibiting processes that consumes ATP [356, 357]. The increases in AMP concentration lead to a conformational change in a subunit of AMPK exposing the ThR-172 phosphorylation site essential for a heterotrimeric complex including LKB1 to phosphorylate and activate AMPK [358].

The activation of AMPK stimulates insulin action in peripheral tissue by stimulating the TSC1:TSC2 complex that in turn inactivates the mTOR/Raptor [359]. Because of its effects on insulin sensitivity, AMPK is a target of several glucose-lowering agents used in diabetes treatment such as metformin and thiazolidenediones [360-362]. AMPK has also an effect on insulin secretion and in controlling the survival of β-cells [363, 364].

1.7.3 LKB1 function in pancreas

Deletion of LKB1 with the RIP2*Cre* or Pdx1*Cre*ER transgenes in β-cells of mice enhances insulin secretion [365]. Mice deleted with the RIP2*Cre* transgene also showed decreased food intake and diminished weight gain [366]. The mechanism of the enhancement of insulin secretion in LKB1 deficient mice is unclear. It has been proposed to be due to a change in the polarity of the β-cells which resulted in an increase in insulin secretion. It has also been proposed that the increase in insulin secretion was due to increased β-cell mass or

insulin content [365, 366]. Deletion of LKB1 also lead to deterioration of mitochondrial structure and function in the β-cells [367].

1.7.4 LKB1 function in the brain

LKB1 has been demonstrated to be involved in cell polarity. Mammalian intestinal epithelial cells that lack LKB1 are unable to polarize [368]. It is, however, possible that LKB1 is involved cell polarity in a cell type-specific manner. In any case, LKB1 has major role in the maintenance of neuronal polarity and axon development in cultured neurons but also in developing cortical neurons in mice [369].

Deletion of LKB1 in POMC-neurons results in animal with impaired glucose tolerance, insulin resistance, defective suppression of glucose production in the liver but no observed effect on body weight [370]. These results suggest a role for LKB1 in the central regulation of peripheral glucose homeostasis. In contrast, mice deleted for LKB1's downstream target AMPK in POMC-neurons suffer from obesity but have normal glucose homeostasis [371].

1.7.5 AMPK's role in the brain

LKB1's downstream target, AMPK, is expressed in the hypothalamus and has been proposed to have a key role in sensing hormones and nutrients to regulate energy homeostasis. Deletion of AMPK in POMC-neurons and AgRP-neurons, individually results in animals suffering from obesity that's due to both reduced energy expenditure and an increase in food intake. Animals remained sensitive to leptin suggesting that AMPK and leptin acts under different pathways in POMC-neurons [371].

Studies have shown that leptin, high glucose and insulin decreased AMPK activity in several hypothalamic regions, whereas ghrelin activates hypothalamic AMPK [372]. Activation of hypothalamic AMPK leads to an increase in food intake in rodents [372]. Mice deficient for AMPK α-subunits in hypothalamus has revealed a role for AMPK in sensing and responding to peripheral hypoglycaemia [373]. In summary, AMPK orexigenic signals activates AMPK and anorexigenic signals inhibits AMPK activation.

1.7.6 RIP2*Cre* neurons

As mentioned above, the arcuate nucleus also contains a third neuronal population intermingled with the POMC/CART and NPY/AGRP-neurons. These neurons do not express POMC or NPY peptides and are thereby distinct from these neurons [229]. RIP2*Cre* neurons were found whilst using transgenic mice harbouring the rat insulin 2 promoter driving *Cre* (RIP2*Cre*) transgene to in addition being expressed in beta cells showed expression in the central nervous system [251].

The two main RIP2 promoters commonly used are a 668bp promoter fragment of the rat insulin promoter named RIP*Cre*^{Mgn} [251] or a 660bp promoter fragment named RIP2*Cre*^{Herr} [374]. Both of these two transgenes drive expression in the brain but with a slightly different expression pattern [193]. RIP2*Cre*^{Mgn} promoter revealed RIP2*Cre* expression throughout the brain and stronger expression in the mid-brain as well as ventral regions. RIP2*Cre*^{Herr} promoter revealed a weaker punctate RIP2*Cre* staining throughout the brain but no apparent regionalization. This is consistent with previous results from Gannon and colleagues [375]. The latter group also reported RIP2*Cre* expression in the spinal cord, neural retina and the posterior pituitary.

Song and colleagues investigated the expression pattern in the RIP2*Cre*^{Mgn} mice with a different reporter mouse. They found expression mainly in hypothalamus but also in the cortex and striatum. In hypothalamus they found RIP2*Cre* expression in SCN, VMH, DMH, mTu and highly concentrated in the arcuate nucleus [376]. This result has also been confirmed with *Cre*-dependent GFP reporter mice [252].

Deletions achieved with the RIP2*Cre* deleter strain have revealed a role of these neurons in energy balance that cannot be accounted for by changes in pancreatic β-cell insulin secretion alone. For example, the deletion of the insulin receptor substrate 2 (IRS2) with the RIP2*Cre* transgene resulted in animals with impaired glucose tolerance and reduced β-cell mass but also hyperphagia, obesity, and increased body length [229, 377, 378]. Choudhury and colleagues also found that insulin and a melanocortin agonist depolarized the RIP2*Cre* neurons, whereas leptin was ineffective [229].

As mention previously in section 1.5.4.2, deletion of the leptin receptor with the RIP2 promoter-drive *Cre* leads to animals with impaired glucose tolerance and obesity but with no effect on food intake [192]. Furthermore, deletion of the transcription factor STAT3 involved in leptin signalling, in β-cells and RIP2*Cre* neurons results in animals with significantly increased body weight, body fat and an increase in leptin levels [379]. Mice with deletion of *Tsc1*, an upstream inhibitory regulator of mammalian target of rapamycin (mTOR), signalling develops hyperphagia and obesity [380].

A few studies have also demonstrated a decrease in bodyweight and hypophagia. Inactivation of fatty acid synthase (FAS) with RIP2*Cre* transgene produces lean and hypophagic mice with an increases metabolic rate and physical activity and defective PPARα signalling in hypothalamus [381]. As mentioned in section above, deletion of the upstream regulator of AMPK the tumour suppressor LKB1 in the RIP2*Cre* neurons and β-cells leads to enhanced insulin secretion and decreased body weight [366]. In addition, these animals suffer from hind-limb dysfunction and axon degeneration at about 7 weeks of age [382]. However, deletion of AMPK with the RIP2*Cre* promoter does not affect bodyweight or food intake [383], suggesting that LKB1 effect on food intake is not via AMPK signalling in the RIP2*Cre* neurons. Furthermore, deletion of LKB1

with the Ins1*Cre* promoter has no effect on food intake or bodyweight demonstrating that it is not LKB1 function in the β-cells that is responsible for the lean phenotype [384].

The RIP2*Cre* neurons are found in several areas of the brain [193, 375, 376] and making it difficult to determine what specific subgroup that is responsible for the energy balance. All of the above mentioned animal models also "suffer" from deleting in the β-cells making it difficult to distinguish what organ is responsible for the phenotype observed.

Therefore, one group did a selective acute ablation of all RIP2*Cre* neurons using the RIP2*Cre*^{Herr} mice bred to animals expressing the DTA toxin receptor upon *Cre* mediated recombination. Intracerebroventricular administration of DTA toxin ablated RIP2*Cre* neurons only, leaving β-cells intact. These animals were found to have a decreased food intake, loss of body weight and fat mass [385].

In another study, Kong and colleagues investigated what specific subpopulation is responsible for the proposed effect of RIP2*Cre* neurons on energy balance. To enable this they deleted the glutamate and GABA synaptic transmitters that are critical components of hypothalamic circuitry in regulation of energy homeostasis [252]. The study found that deletion of glutamate in RIP2*Cre* neurons had no effect but that deletion of GABA in RIP2*Cre* neurons lead to animals that had a modest increase in body weight but normal food intake due to reduced energy expenditure. The animals also became exceptionally sensitive to high-fat diet-induced obesity, due to altered diet-induced thermogenesis.

This group also selectively stimulated the RIP2*Cre* neurons in the arcuate nucleus and observed that this area mediated the effect on energy expenditure without affecting food intake. The group further examined the RIP2*Cre* neurons by identify downstream neurons receiving input from the arcuate RIP2*Cre* neurons and found that RIP2*Cre* neurons mainly projected into the PVN [252].

1.8 Study of gene function using transgenic mice

Mouse models for human disease are important for studying the function of genes. A transgenic mouse is a mouse with its genome genetically modified. Early studies using mice as models to study gene functions came from spontaneous mutations arising in mouse colonies. This approach of studying genetics is referred to as "forward genetics". Now, gene transfer technologies are being used as the main approach studying gene functions. This method allows the generation of novel genetic alterations in the genome. The study of a specific engineered gene alteration is called reversed genetics.

The first gene transfer technology for the generation of transgenic mice used was the injection of DNA into fertilized oocytes. This approach led to random integration in the genome in terms of copy number and

integration site [386]. This method was replaced with targeted integration by homologous recombination [387].

A third method, revolutionizing the way to genetically engineer mice to study gene function is the introduction of the *Cre*-loxP recombination system. With this system recombination can occur only in cells expressing *Cre*-recombinase, thereby restricting the gene alterations to selected tissues [388-390]. Examples of this approach, including the use of RIP2 *Cre*s, have been introduced in preceding sections.

1.8.1 *Cre*-loxP recombination system

The *Cre*-loxP recombination system is one of the tools most used to genetically engineer mice. The site-specific recombinase technology is used to delete, insert, translocate, also inversion of defined sites in the DNA. The system consist of the bacteriophage P1-derived integrase *Cre* recombinase that specifically binds to loxP (locus of crossover in P1) sequences and catalyzes recombination between the sites [391]. The loxP site comprises of two 13bp inverted repeats with an asymmetric 8-bp spacer sequence. The specific orientation of the loxP sites relatively to each other determines the result of the *Cre*-mediated recombination. If a DNA sequence is flanked with two loxP sites that are directly repeated, the flanked DNA sequence is cut out as a circular DNA irreversibly. However, if the DNA is flanked by two oppositely oriented loxP sites the DNA will be inverted.

This method, in combination with gene targeting techniques, enables removal of loxP-flanked (floxed) selection sites introduced by gene targeting in the mouse genome. Using this method, gene alterations that are tissue-, cell type- or developmental stage-specific can be achieved depending on available *Cre*-driver transgenic animals. The cell specificity is dependent on where the promoter driving *Cre* is active.

Cre recombinase activity can be detected with a "reporter" construct that is under the control of a ubiquitously expressed promoter. The reporter gene consists of a floxed transcriptional stopper that is followed by detectable gene such as bacterial β-galactosidase or fluorescent proteins. Upon *Cre* expression the floxed transcriptional stopper is irreversible excised allowing transcription of the detectable gene [392, 393] or variations of this method [394].

1.8.2 Pancreatic *Cre*-driver lines

The development of mouse lines expressing *Cre* in pancreas or specifically in pancreatic β-cells has helped the discovery and *in vivo* analysis of many important genes involved in the regulation of glucose homeostasis. Several promoters driving *Cre* in different cells of pancreas has been developed. The type of

promoter to express *Cre* recombinase in pancreatic cells is an important choice to determine during what development stage and the location the gene is active [395].

Expressing *Cre* under a fragment of the of rat insulin 2 promoter [251, 374, 396] or from the pancreas duodenal homeobox 1 (Pdx1) [196, 397] is an approach that has been well used and characterized to drive expression in β-cells. However, these promoter fragments also drive expression in other areas outside the β-cell [193].The Pdx1 gene is expressed in the pre-pancreatic endoderm and after development becomes more selective in β-cells and lower levels in other endocrine cells. Therefore, the Pdx1-*Cre* drivers deletes in all pancreatic cells. However, when the Pdx1 promoter is driving a *Cre*ER a tamoxiofen activated *Cre*, only expression and subsequent recombination is seen in islets and acinar cells [196]. Besides expression in pancreas the Pdx1 promoter also drive expression in duodendum, antral stomach, bile duct as well as in hypothalamus and inner ear [193, 398, 399].

The more β-cell-specific promoters that utilize RIP2 gene expression are selectively expressed in 80% or more of the β-cells. On the downside, this promoter is "leaky", with expression in the brain areas such as hypothalamus and the pituitary gland [193, 376]. Therefore, transgenic mice expressing the *Cre* recombinase selectively in the β-cells under the control of insulin 1 promoter have now been developed by knockin of *Cre* at the Ins1 locus [400] (see below).

Several other promoters such as *Neurog3*, *Nkx2.2*, *Pax4*, *Pax6* or *Isl-1,*expressed at different developmental stages of the β-cell, have been used. However these also drive *Cre* expression in islets in non- β-cells, as reviewed by [395].

Relatively fewer *Cre*-drivers have been developed for other endocrine cell types. For α-cells, two different rat glucagon promoter have been developed that cause *Cre*–mediated recombination in glucagon expressing cells [374, 401]. However, there have been reports that the *Cre* deleter strains are rather inefficient [100, 308, 402, 403]. However, a new alternative glucagon promoter driven *Cre* with stronger expression, iGlu*Cre* has been developed [404].

1.8.3 Ins1*Cre*-driver

To overcome the limitations with the "leaky" Rip2 promoter Thorens and colleagues have generated a transgenic mouse strain with selective -cell expression utilizing the *Ins1* locus [400]. The *Ins1* gene in contrast to Ins 2 gene expressed selectively in β-cells [25, 26]**.** Therefore, this transgenic mouse lacks expression in hypothalamus and other brain areas. Thorens and colleagues verified that no ectopic expression is found by crossing the Ins1*Cre* to a *Cre* reporter strain demonstrating no Ins1*Cre* expression in

hypothalamus, cortex or cerebellum. In addition, animals containing the Ins1*Cre* transgene had normal body weight gain, glycaemic levels and glucose tolerance [400].

The transgene is "knocked-in" by homologous recombination at the second locus of the *Ins1* gene so that *Cre* coding sequences start at the initiation codon and replaces the *Ins1* coding sequence. Inserting the *Cre* recombinase behind its endogenous promoter reduces the incidence of ectopic expression, in contrast to using predefined promoter fragments such as with the Rip2 promoter. The reason is that the knocked in transgene can use all of the regulatory sequences not exclusively encoded in promoter fragment [400].

1.8.4 iGLUi*Cre*-driver

The mouse strain iGLUi*Cre* contains a glucagon promoter-driven *Cre* with a region upstream of the proglucagon gene [404]. These animals were created using a construct where the proglucagon start codon in exon 2 was replaced with an improved version of *Cre* recombinase *iCre* [405]. The transgene construct was integrated randomly into the mouse genome and the animal's transgene copy number was estimated to be 2-3.

Apart from expression in glucagon-expressing α-cells this strain also drives *Cre* expression in other sites of proglucagon expression such as the NTS [406] and in intestinal L-cells [404].

1.8.5 *Cre*-inducible ROSA26-tdRFP transgenic mouse

The *Cre*-inducible reporter mouse strain contains a tandem-dimer red fluorescent protein (tdRFP) with a strong brightness, enabling detection of *Cre*-expressing cell [394]. The tdRFP cassette is inserted in anti-sense location at the ROSA26 locus preceded by "transcriptional-stop" cassette and flanked with oppositely loxP sites. The cassettes loxP sites are combined with mutant loxP2272 that are both recognized by *Cre* recombinase but do not allow for the subsequent recombination step when combined with the WT loxP sites. The two mutant loxP2272 sites are inserted around the reversed tdRFP cassette as indicated in figure 1.8 and recombination will only occur when the loxP sites are identical.

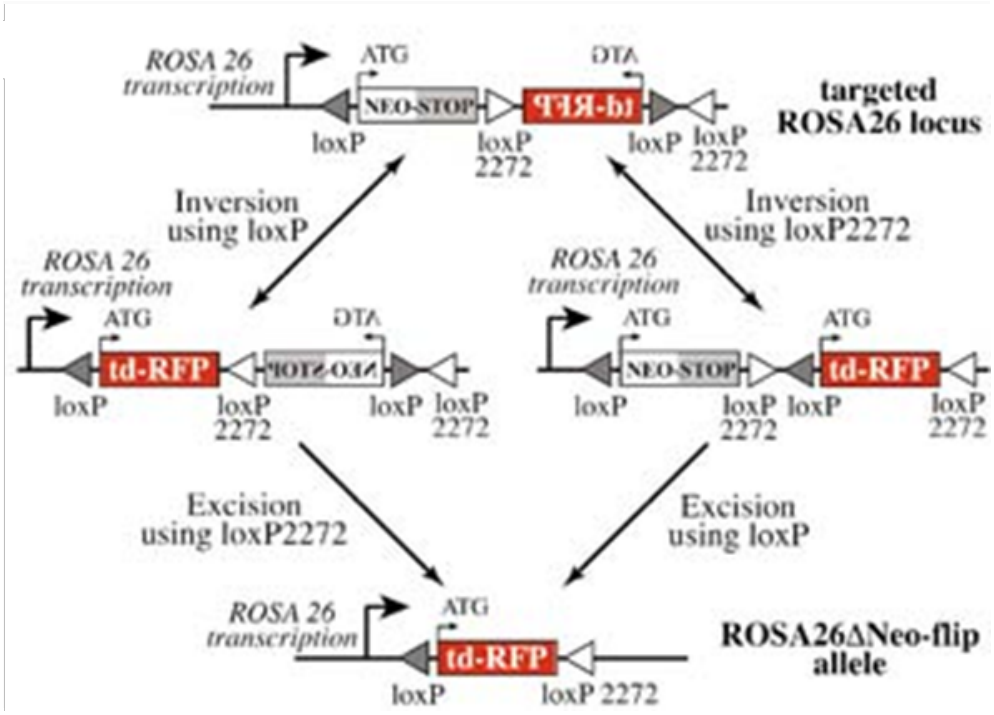


Figure 1.7 - *Cre*-inducible expression of tdRFP. The ROSA26 locus containing the neomycin-tdRFP cassette. The tdRFP cassette is inserted in inverse orientation and flanked by two oppositely oriented wild-type loxP sites (filled triangles). Two additional, mutant loxP2272 sites (open triangles) inserted directly repeated, embracing the tdRFP cassette in addition to the wild-type loxP sites. Middle: Schematic representation of the two alternative recombination intermediates generated by *Cre*-mediated inversion at the loxP and loxP2272 sites, respectively. The alternative intermediates are reversible. Bottom: Final recombination step excision of the transcriptional stop cassette. After inversion of tdRFP and removal of the "Neo-STOP" cassette, tdRFP is expressed by the ROSA26 locus. The remaining loxP sites (wild type and mutant) are incompatible and therefore no further recombination can occur. Picture from [394]

During the *Cre* mediated recombination the intervening sequences are inversed either by using the matching *lox*P sites or the matching loxP2272 sites. In a last recombination step the "stopper" cassette is excised irreversibly along with its loxP or loxP2272. This creates a "leak-proof" *Cre*- mediated expression of tdRFP [394].

1.9 Study of gene function using viral gene-mediated transfer into the brain

Until recently, gene expression in neurons could only be altered by the use of transgenic animals or by microinjection of neurons in culture. Now, several recombinant viral vector system are being utilized to transduce neurons and mediate gene transfer [407]. Using these techniques, adeno-associated viruses are intracranially injected into live animals using stereotactic coordinates for precise delivery of the virus. Depending on the virus and its promoter driving the gene expression groups of neurons in different regions can be targeted.

1.9.1 Adeno-associated viruses

Adeno-associated viruses (AAV) were firstly discovered in 1960s, as a replication deficient virus belonging to the family of Parvoviridiae. AAVs are able to transduce both non-dividing and dividing cells and have a long-term gene expression and are therefore regularly utilized as a gene delivery vector. In addition, the wild-type virus has no pathogenicity and is therefore a safe option.

The AAV virus particle is a small - approximately 22 nm - non-enveloped and replication-deficient virus with an icosahedral capsid composed of three viral proteins. It is packaged as a linear single stranded DNA genome of 4.7 kB [408].

The genome is flanked on each side with inverted terminal repeats (ITRs) comprising of 145 base pairs that are required for the efficient multiplication of the virus. The distal 125 bases of the ITRs are palindromic forming a hairpin enabling initiation of DNA synthesis [409]. The ITRs flank two genes *Rep* (replication) and *Cap* (capsid). *Rep* encodes four regulatory proteins involved in genome replication. The *Cap* gene encodes three capsid proteins that assemble into the icosahedral capsid [410, 411].

The ITRs are also essential for AAV virus encapsulations virus genome contains Rep binding elements (RBEs) necessary for replication as well as the terminal resolution site (TRS) located in the ITRs. These elements are used by the viral regulatory protein (Rep) to process double-stranded intermediates. After internalization into cells, the virus either follows the lytic or lysogenic pathway. The lytic pathway only occurs if the virus is co-infected with helper viruses such as adenovirus [412]. Upon infection by AAV on its own the virus genome integrates into a region on chromosome 19 with the help of the rep proteins [413].

1.9.2 Adeno-associated virus as vector

The single stranded AAV genome has been cloned into a plasmid containing a double stranded copy of the genome. The wild type genome can be rescued from this plasmid and packaged into virus particles and able to infect cells [414, 415].

The utilization of AAV as vectors for delivering of transgenes was firstly established by Hermonat and Muzyczka in 1984 [416]. The capsid proteins are added by co-transfection of “helper” plasmids. Constructs developed later and the one used in this thesis contains only the two ITRs required in the vector for rescue, replication and packaging removing both the Cap and Rep genes. All other elements need for efficient virus production are provided by the helper plasmids [417].

There are several serotypes AAV with different tropism. AAV serotype 2 (AAV2) have the ability to efficiently transduce neurons but not glial cells [418-420]. AAV2 enters cells via attachment to the heparan sulphate proteoglycans and a co-receptors αvβ5 integrin [421].

1.9.3 Delivery of AAV into the mouse brain with stereotactic surgery

Stereotactic surgery is a technique to localize and target specific structures in the brain of living animals. This method allows for high precision delivery of substances as well as the ability to introduce substances that are restricted by the blood brain barrier. By injecting genetically engineered viruses such as AAV specific areas of the brain can be targeted and gene expression altered.

Aims of this thesis.

The aims of this thesis are to:

1. Investigate the role of leptin receptor signalling in pancreatic β-cells on glucose homeostasis.

2. To investigate the role of leptin receptor signalling in GLP-1 expressing neurons and pancreatic α-cells on body weight progression and glucose homeostasis.

3. To explore the role of the LKB1 in RIP2Cre neurons of the arcuate nucleus of hypothalamus on body weight and glucose homeostasis.

Chapter 2 Materials and Methods

2.1 *In vivo* animal work

2.1.1 Generation of animals lacking LepRb signalling specifically in β-cell

Animals harbouring LepRb^{F} alleles [195] on a Friend Virus B (FVB) background were kindly provided by Dr Streamson Chua (Columbia University) and twice backcrossed with wild type C57BL/6 mice (Charles River,UK). Heterozygous LepRb$^{F/+}$ mice were then mated to heterozygote Ins1*Cre* animals on a C57BL/6 background [422] generating animals double heterozygous for Ins1*Cre*$^{+/-}$ and LepRb$^{F/+}$. Double heterozygotes were then bred to homozygote LepRb$^{F/F}$ to generate Ins1*Cre*LepRb^{KO}, creating animals deleted for the leptin receptor in β-cells. Animals were born with normal mendelian ratio and were kept on a mixed background of FVB and C57BL/6.

2.1.2 Generation of mice lacking LepRb signaling in pancreatic alpha cells and Glp-1 expressing cells

The LepRb *flox'd* mouse was crossed with an iGlu*iCre* animal [404] on C57BL/6 background also containing a *Cre*-reporter transgene Rosa26.tdRFP [394] enabling detection of efficient *Cre*-mediated recombination. Triple heterozygotes iGlu*iCre*LepRb$^{F/+}$ crossed again with LepRb$^{F/F}$ generating iGlu*iCre*LepRb^{KO}. Animals were born with normal Mendelian ratio and were kept on a mixed background of FVB and C57BL/6.

2.1.3 Mice *flox'd* for LKB1 (LKB1$^{F/F}$)

Mice harbouring flox'd alleles of exon 3-6 of the LKB1 gene (Mouse Models of Human Cancer Consortium. http://mouse.ncifcrf.gov/) were kept homozygous on a C57BL/6 (Charles River,UK) background.

2.1.4 Genotyping

2.1.4.1 DNA isolation from ear-notches

Genomic DNA was extracted from ear clips using the Proteinase K method. The following reagents 5 µl of 10x PCR buffer (Thermo Fisher Scientificr), 1.25µl proteinase K (Invitrogen) 43.75 µl nuclease-free H_2O (Qiagen, Hilden, Germany), was added to the ear notch. The mixture was incubated in a thermal cycler (2720 thermal cycler, Thermo Fisher Scientific) for 40 min at 55°C and finally the proteinase K was heat inactivated for 10 min at 95°C.

2.1.4.2 PCR screening *iCre* and tdRFP

For PCR amplification of *iCre*, tdRFP and β-catenin (tissue control) 3 µl of the extracted DNA was used as template in a 25 µl PCR reaction, comprising of: 12.5 µl GoTaq green master mix (Promega, UK), primers 1 µM each (primer sequences are given in table 2.1), 1.25µl DMSO and 5.75 µl of nuclease free water. Pre-heating step 1: 5 min 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 57°C, 1 min 72°C and followed by a final extension step for 5 min at 72°C.

Table 2.1 Primers sequences genotyping *iCre* and tdRFP

Target	Forward primer sequence 5'-3'	Reverse primer sequence 5'-3'
iCre	Cre002: gacaggcaggccttctctgaa	Cre003: cttctccacaccagctgtgga
β-catenin	RM41: aaggtagagtgatgaaagttgtt	RM42: caccatgtcctctgtctattc
tdRFP	Anti: ctacaggaacaggtggtgg	Sense: ctgttcctggggcatggc

2.1.4.3 PCR screening Ins1*Cre* and LepRb

For genotyping of Ins1*Cre* (primer sequences are given in table 2.2), the PCR reaction was performed in a total of 20µl mixture comprising of: 5x Phire^{TM} reaction buffer, 0.2 mM dNTP (Qiagen, Hilden, Germany), 0.5 µM forward and reverse primers, 0.4 µl Phire^{TM} hot start DNA polymerase (NEB, Hitchin, UK) and 1µl DNA. The reaction consists of a 30s 98°C pre-heating step, followed by an amplification step of 30 cycles: 98°C for 5 seconds, T_m 60°C for 5 seconds and 72°C for 10 seconds. After the last cycle, a prolonged extension step was carried out for 1 min. at 72°C.

Table 2.2 Primer sequences genotyping Ins1*Cre* and LepRb

Target	Forward primer sequence 5'-3'	Reverse primer sequence 5'-3'
Tissue control	FER2-Q2: accttcagaccttggcgttggagg	FER2-R2: cctgaggttcctgttgctgtgactcc
Ins1*Cre*	*Cre* jva rv 2: gccagattacgtatatcctggcag	*Cre* jva fw 2: ttcactggttatgcggcgg
LepRb^{F}	104: cagccgaccaatgcttatt	105: acaggcttgagaacatgaacac

2.1.4.3 PCR screening for LKB1

The PCR reaction was performed in a total of 20 µl reaction mixture, comprising of 5X Phire reaction buffer, 0.2 mM dNTPs (Qiagen, Hilden, Germany), 0.4 µl Phire hot start DNA polymerase (NEB, Hitchin, UK), 0.5 µM forward and reverse primers (sequences are as shown in table 2.3). Pre-heating step for 30 secs at 98°C, followed by 30 cycles of 5 secs at 98°C, 5 secs at 57°C, 20 secs at 72°C. Lastly, a final extension step for 1 min at 72°C.

Table 2.3 Primer sequences genotyping LKB1

Target	Forward primer sequence 5'-3'	Reverse primer sequence 5'-3'
LKB1^{F}	gggcttccacctggtgccagcctgt	gagatgggtaccaggagttggggc

2.1.5 Animal maintenance

Animals were maintained in individually ventilated cages in a pathogen-free environment in a 12h light/dark cycle. Animals had free access to standard mouse chow (research Diet, New Brunswick, NJ, USA). All animal procedures were performed at the Imperial College Central Biomedical Service and were approved by the U.K. Home Office and were compliant with the Animals (Scientific Procedures) Act 1986 of the United Kingdom.

2.1.6 Body weight

Body weight was followed from the age of weaning (3-4 weeks) to the age of 15 weeks or 30 weeks. Animals had free access to food and body weight was recorded weekly.

2.1.7 Intraperitoneal glucose tolerance test (IPGTT)

Intraperitoneal glucose tolerance tests were performed on mice fasted overnight for 16 h. Mice were injected intraperitoneally (IP) with 1g D-glucose from SigmaAldrich (Dorset, UK) per kg mouse weight. Blood samples were taken from the tail vein at time points 0, 15, 30, 60 and 120 min after IP glucose. Blood glucose levels were measured using a handheld automated glucometer (Accucheck, Roche, Burgess Hill, UK).

2.1.8. Intraperitoneal insulin tolerance test (ITT)

Animals subjected to intraperitoneal insulin tolerance test were fasted for 5hrs and intraperitoneally injected with bovine insulin from SigmaAldrich, (Dorset, UK) using 0.75U per kg animal weight. Blood glucose levels were measured from the tail vein at 0, 15, 30 and 60 min post injection.

2.1.9 *In vivo* glucose-stimulated insulin secretion (GSIS) and glucagon secretion

For plasma insulin measurements, animals were fasted for 16 hrs O/N. Animals were challenged with 3g glucose per kg mouse by intraperitoneally injection. For plasma glucagon measurement animals were fasted O/N and injected with bovine insulin from SigmaAldrich, (Dorset, UK) using 0.75U per kg animal weight. Blood samples (20-40 µl were taken from the tail vein at time point 0, 15 and 30 min post injection. Blood was collected in heparin coated tubes (Sarstedt, Beaumont Leys, UK). Blood plasma was obtained by centrifugation at 13,200 rpm for 20 min at 4°C.

2.1.10 Isolation of pancreatic islet from mice

Animals were killed by dislocation of the neck. The pancreas was distended by injection of 5ml NB8 collagenase (1mg/ml in RPMI-1640); (SERVA, Heidelberg, Germany) into the common bile duct clamped at the duodenal entrance. The distended pancreas was excised and placed on ice in 2 ml collagenase (1mg/ml in RPMI-1640). The isolated pancreas was digested for 10 min in a 37°C water bath. The digested pancreas was washed 3 times with RPMI-1640. Islets were separated by a histopaque density gradient centrifugation method (SigmaAldrich, Dorset, UK). Densities were 1.119 g/ml, 1.083 g/ml and 1.077 g/ml added to 1:1:1 mixture. Gradients were centrifuged at 2500 RPM for 20 min. Islets were handpicked after incubation at 1-2 hrs at 37°C into RPMI-1640 medium supplemented with 10% fetal calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin. Islets were cultured for 24-36 h before further analysis.

2.2 *In vitro* assays

2.2.1 Insulin secretion from isolated islets

Isolated size-matched pancreatic islets were pre-incubated in Krebs-Ringer bicarbonate Hepes (KRBH) buffer compromising (mM): 120 NaCl, 4.8 KCl, 24 $NaHCO_3$, 0.5 Na_2HPO_4, 5 HEPES, 2.5 $CaCl_2$, 1.2 $MgCl_2$ and 5 D-glucose, pH 7.4) complemented with 0.1% BSA and 3 mmol/l glucose at 37°C and shaking at 120 spm. The islets were further incubated for 30 min at 37°C in 0.5 ml of KRBH-buffer in the presence of either 3 mM glucose, 16.7 mM glucose or 20 mM KCl. After final incubation the islets were collected and lysed in 1 ml acidified-ethanol (75% [vol./vol.] ethanol, 1M HCl, 0.1% [vol./vol.] Triton X-100) and sonicated for 10

secs and stored at -20°C until measurement of total insulin. For measurement of secreted insulin, the medium was collected and stored at -20°C until further analysis.

2.2.2 Glucagon secretion from isolated islets

Batches of 20 size matched islets were pre-incubated for 30 min at 37°C in KRBH-buffer as described above but supplemented with 10 mM glucose. Islets were further incubated in 250 µl KRBH-buffer supplemented with either 0.5 mM glucose, 10 mM glucose or 0.5 mM glucose supplemented with10 nM leptin at 37°C and shaking at 120 spm. At the end of the incubation the medium was collected and whole islets collected as described above and stored at -20°C until further analysis.

2.2.3 Insulin and glucagon measurement

Secreted and total hormone levels were measured by homogeneous time-resolved fluorescence-based (HTRF) assay from Cisbio (Bagnols-sur-Ceze, France) according to manufactures instructions.

2.2.4 Plasma insulin and plasma glucagon measurement

Blood plasma insulin and glucagon levels were measured with ELISA kits from CisBio (Bagnols-sur-Ceze, France) and Mercodia (Uppsala, Sweden), respectively, according to the manufactures instruction.

2.2.5 Intracellular free Ca^{2+} imaging

Isolated islets were incubated (37°C, 95% O_2/5% CO_2) for 30 min with Fluo2-AM 10µM (Teflab) diluted with DMSO (0.01%, wt/vol) and pluronic acid (0.001%, wt/vol; Invitrogen) in bicarbonate buffer (120 mM NaCl, 4.8 mM KCl, 1.25 mM NaH_2PO_4, 24 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 3 mM D-glucose). Islets were allowed to acclimatize to the 3mM glucose concentration and imaging was performed at 34-36°C on a glass coverslip aluminium chamber (Digital Pixel).

Signals from intracellular Ca^{2+} loaded with Fluo2-AM were captured with a Nipkow spinning disk head (Yokogawa CSU-10), the spinning disk allows for islets to be recorded for long periods with minimal phototoxicity. The spinning disk was coupled to a Zeiss Axiovert M200 microscope fitted with x10–x20/0.3–0.5 numerical aperture (NA) objectives (EC Plan-Neofluar, Zeiss). Islets were illuminated at 491 nm using a solid state laser (Cobalt) with emitted light detected from 500-550 nm using a 16-bit back-illuminated EM-CCD (Hamamatsu C9100-13). To provide an interface the software Volocity™ was used (PerkinElmer).

Individual cells (loaded with fluo-2) were identified and the intensity over time traces measured. Glucose-responsive cells were manually identified by increase in the $[Ca^{2+}]_I$ in response to glucose. The intracellular free Ca^{2+} signals were normalised using the function F/F_{min} where F represents the fluorescent at a certain time point and F_{min} is the minimum of fluorescence recorded.

2.2.6 Connectivity analysis

Connectivity analysis was performed as described [423]. Individual cells (loaded with fluo-2) were localised by using a region of interest (ROI), and the intensity over time traces. Numerical coordinates (X-Y) were extracted using Cartesian coordinate system and the resulting data was imported to R-project R - development core team and IgorPro (Wavemetrics Lake Oswego, OR). All possible cell pair combinations were evaluated by Pearson product-moment correlation coefficient (Pearson R). The mean percentage of significantly correlated cell pairs was displayed in a graph at the low glucose concentration (G3) and the transition from G3 to high glucose (G11).

Corresponding maps of significantly correlated cell pairs were assembled by converting the normalized intensity to a value (1-100%).

2.3 Immunohistochemistry of pancreas and brain

2.3.1 tdRFP and glucagon staining

Animal were transcardially perfused-fixed with 4% PFA (as described in more detail in 2.5.4). The pancreas was excised, post-fixed for 1 h and transferred into a tissue processing cassette. Fixed pancreas was subsequently embedded in paraffin wax and sectioned at 5 µm and mounted on superfrost slides.

Slides containing pancreas were submerged in histoclear (Sigma Aldrich, Dorset, UK) for 20 mins. Slides were then washed with decreasing concentrations of ethanol (100%, 90% and 70%) and finally water. An antigen retrieval step was performed by treating slides with vector antigen solution (Vector Laboratories). The slides were subsequently blocked in 0.25% [vol./vol.] goat/donkey serum (Dako, UK) supplemented with 2%bovine serum albumin (BSA) with 0.1% [vol./vol.] tween and incubated for 20 mins at room temperature. Sections were labelled with anti-RFP (Rockland, 600-401-379) in 1:50 dilution and anti-glucagon (Dako, UK) 1:500 dilution and incubated in a moist box at 4°C over night.

Slides were washed three times in phosphate-buffered saline (PBS) comprising of 13.7 mM NaCl, 0.27 mM KCl, 1mM Na_2HPO_4, 0.2 mM KH_2PO_4 complemented with 0.25% BSA and 0.25% Triton X-100 followed by incubation for 2 hrs in the dark with secondary antibodies (secondary-Alexa 568, 1:250 dilution)

and anti-glucagon (secondary-Alexa 488 1:250 dilution). After a final wash in PBS slides were sealed using Vectashield hardset mounting medium with DAPI (Vector Laboratories).

Fluorophores were excited using a Zeiss Observer Vivatome and a 20x/NA 0.8 x objective and 365 nm, 470-nm and 540-580-nm LEDs. Emission was detected using Semrock filters centred on 377/50 nm, 472/30 nm, and 534/20 nm for DAPI, Alexa 488, and Alexa 568, respectively.

2.3.2 Insulin and glucagon staining

Mice were killed by cervical dislocation and pancreata were extracted and fixed in 10% (v/v) neutral buffered formalin at 4°C for 18 h. Embedding, sectioning and the subsequent treatment before staining of the slides were performed as described above.

Sections were labelled with polyclonal anti-insulin (Dako A4056, 1:200 dilution, Secondary-Alexa 488 1:1,000) and anti-glucagon (Sigma, G2654 or Santa Cruz, sc-13091, 1:200 dilution, Secondary-Alexa 568 1:500) antibodies and sealed using Vectashield hardset mounting medium with DAPI (Vector Laboratories).

Fluorophores were excited as described above.

2.3.3 Stat3 phosphorylation staining in whole islets

Isolated whole islets were incubated for 30 min in Kreb's Ringer bicarbonate buffer followed by incubation with either leptin (10 nM) or IL1-β (50 ng/mL) and TNF-α (1000 pg/mL) or control with no additions at 37°C and shaking 120 spm. Islets were washed in PBS and fixed in 4% PFA for 1 h at 4°C.

Antigen retrieval steps were performed by incubating islets in 0.01 M Tris-HCl for 5 min at 90°C, before cooling to room temperature and incubation in 100% methanol at -20°C for 10 min. Islets were blocked with 0.1% Triton X-100 and 10% normal goat serum diluted in 0.1M PBS for 1 h at room temperature.

Free-floating islets were stained immunohistochemically with anti-pSTAT3 (pTyr-705; NEB, 4113S, 1:100 dilution, secondary-Alexa 488 1:500) and anti-insulin (1:200 dilution, secondary-Alexa 568 1:500) antibodies, and mounted onto glass slides, as above. Images were obtained by using a Zeiss LSM-780 microscope and a 60x/NA 0.8 x objective or using a Zeiss Observer Vivatome.

2.3.4 tdRFP and Stat3 phosphorylation staining in brain

Animals were fasted O/N and IP injected with leptin (5mg/kg) or saline control and sacrificed 40 min later. Animals were transcardially perfused-fixed with 0.1 M PB followed by 4% PFA. Brains were taken out and further fixed in 4% PFA for 1 h at 4°C and cryoprotected in sucrose. Brains were sectioned on a freezing microtome (30μm) and collected in Eppendorf tubes containing 0.1 M PB and stored at 4°C until further used.

Antigen retrieval steps were performed as described above. Free-floating brain sections were blocked with 0.1% Triton X-100 and 10% normal goat serum diluted in 0.1M PB for 1 h at room temperature. STAT3 phosphorylation in neurons was detected with antibodies raised against pSTAT3 (pTyr-705; NEB, 4113S). These were added to the blocking solution in a 1:100 dilution and incubated for 72hrs at 4 °C. Upon co-staining with tdRFP, after 60 hrs anti-dsRED (Clontech #632496) was added to the solution at a 1:1000 dilution and incubated overnight.

Subsequently, brain sections were washed 3 times for 5 min in 0.1M PB at room temperature, followed by incubation with a secondary-Alexa 488 (1:500 dilution) and for visualizing anti-dsRED secondary-Alexa 568 was added (1:500), for 2 h in the dark at room temperature. Brain slices were washed and mounted onto polylysine-coated slides (VWR), shortly air dried and sealed with Vectashield mounting medium with DAPI (Vector Labs). Images were obtained by using a Zeiss LSM-780 microscope and a 60x/NA 0.8 x objective.

2.4 Gene expression analysis

2.4.1 RNA extraction and reverse transcription

Total RNA from mice islets or brain tissue was extracted by incubation in Trizol reagent (Invitrogen, Paisley, UK) for 10 min. The RNA were incubated in 0.2 volume of chloroform (Sigma, UK) followed by centrifugation at 12000 rpm at for 4°C for 15 min. The supernatant was carefully removed and 2 μl of glycogen was added followed by the addition of 0.8 volume isopropanol and incubation for 15 min at 4°C. The mixture was centrifuged 12000 rpm and the supernatant removed and the pellet was washed in 70% ethanol and centrifuged at 12000 rpm. This step was repeated twice.

FACS sorted islet cells (FACS isolation was performed by Dr Alice Adrianssens and described in appendix 1) the amount of 150 ng for β-cells, 70 ng for α-cells and 40 ng for δ-cells RNA were reversed transcribed to cDNA. For hypothalamic RNA 40 ng were reversed transcribed to cDNA. Superscript III (Life technologies) reverse transcriptase was used to synthesize cDNA in a total of 10μl reaction mixture. The PCR mix was heated at 25°C for 10 mins, 37°C for 2 hours and 85°C for 5 mins.

2.4.2 Quantitative Real-Time PCR

Analysis was performed with a 7900 HT Fast Real-Time PCR system (Applied Biosystems). The PCR reaction mix consisted of 20 ng first-strand cDNA template, 0.35µmol/l primer pairs for LepRb, and 6µl PCR Master mix (Invitrogen, Paisley, UK). Primers were tested with RNA extracted from mouse hypothalamus serially diluted to ensure efficient amplification of the target sequence. Expression of selected targets was compared with that of *Cyclophilin* measured from the same sample in parallel on the same plate.

The PCR reaction mixture was preheated to 60°C for 2 mins followed by 95°C for 10 mins. PCR amplification was performed for 40 cycles of 95°C for 15 seconds and 60°C for 1 min, followed by a dissociation step consisting of 95°C for 15 seconds, 60°C for 1 min and lastly 95°C for 15 seconds.

Table 2.4 Primer sequences quantitative RT-PCR

Target	Forward primer sequence 5'-3'	Reverse primer sequence 5'-3'
LepRb	gtttcaccaaagatgctatcga	gagcagtaggacacaagagg
Cyclophilin	aagactgagtggttggatgg	atggtgatcttcttgctggt

2.3.3. Genomic DNA extraction and amplification of the excised locus

Genomic DNA was harvested from Ins1*Cre*LepRb^{KO} and control animals LepRb$^{F/F}$ from hypothalamus, islet and spleen and from iGlui*Cre*LepRb^{KO} and control LepRb$^{F/F}$ from olfactory bulb, gut, islet, liver and hypothalamus. Harvested tissues were stored at -20°C until genomic DNA was extracted with a kit from SigmaAldrich (Dorset,UK). Extracted DNA (100ng) was used as template for PCR amplification. The PCR reaction mixture consisted of: 5x Phire^{TM} reaction buffer, 0.2 mM dNTP (Qiagen, Hilden, Germany), 0.5 µM forward and reverse primers, 0.4 µl Phire^{TM} hot start DNA polymerase (NEB, Hitchin, UK) in a total of 20µl. Three sets of primers were used for Ins1*Cre*LepRb^{KO} and control animals LepRb$^{F/F}$ as indicated from table 2.5. For DNA from iGlui*Cre*LepRb^{KO} and LepRb$^{F/F}$ only primer 104 and 105 were used. Pre-heating step for 30 secs at 98°C, followed by 30 cycles of 5 secs at 98°C, 5 secs at 57°C, 3-4 secs at 72°C. Lastly, a final extension step for 20 sec at 72°C.

Table 2.5 Primer sequences for excised DNA		
Target	Forward primer sequence 5'-3'	Reverse primer sequence 5'-3'
LepRb^{F}	103: tgagttccctcatgattctgg	
LepRb^{F}	104: cagccgaccaatgcttatt	105: acaggcttgagaacatgaacac

PCR amplicons was subjected to gel electrophoresis on a 1.5% agarose gel (SigmaAldrich, Dorset, UK) and the DNA was visualised with ethidium bromide.

2.5 Injection of AAV with stereotactic surgery

2.5.1 Adeno associated virus 2 (AAV2)

Two different AAV were purchased from Vector BioLabs (Philadelphia, US) adeno associated virus serotype 2- mouse insulin 2 promoter – enhanced green fluorescent protein -Thoseaasigna virus 2A (AAV2-mIP2-EGFP-T2A-*iCre*) and control virus AAV2-mIP2-EGFP-T2A without *Cre* recombinase. The virus contains an expression cassette consisting of a 1.1kB fragment from the mouse insulin 2 promoter (mIP2) driving eGFP-T2A-*iCre* construct. The T2A fragment in the constructs allows for discrete production of a protein product *Cre* recombinase in this case. The *Cre* recombinase here is a codon-optimized version leading to a higher level of *Cre* protein expression (*iCre*). The virus titers are given in genome copies per mL (GC). The titer for AAV2-mIP2-EGFP-T2A-*iCre* was $2.1x10^{13}$GC per ml and for AAV2-mIP2-EGFP-T2A was $2.3x10^{13}$GC per ml. Viruses were aliquoted and stored at -80°C until used.

2.5.2 Optimization of stereotactic coordinates

Initial stereotaxic coordinates of the ARC were derived from The Mouse Brain in Stereotaxic Coordinates (Academic Press). Coordinates are given relative to bregma in a three-dimensional (X,Y and Z) grid in mm. Bregma is the intersection of the coronal and sagittal sutures on the skull (figure 2.1). The y plane represents the front to back or anterior-posterior (AP), x-plane represents left and right to bregma or the medial to lateral (ML), the z-plane represents the dorsal to ventral (DV) distance relatively to the skull surface.

Co-ordinates were optimized by stereotactic injection of indian ink (as described in the next section 2.5.3) into the hypothalamic ARC of mice cadaver. To target both sides of the ARC bilateral injections were performed.

Freshly killed mice (by overdose of pentobarbital) were mounted onto a stereotactic frame (KOPF, US), and the skull immobilized by using tooth bar and aural positioning studs.

The skull was exposed to visualize bregma, lambda and sagittal and coronal sutures by making a surgical anterior posterior incision with a scalpel blade and drying the skull with a cotton pad. The skull was adjusted to level bregma with lambda by positioning the head by adjusting the nose bar level as well as rearranging the aural studs. Bilateral holes (1mm in diameter) were drilled through the surface of the skull using a hand-held drill (World Precision Instruments Inc).

A 26 gauge guide cannula was inserted followed by a 33 gauge internal cannula (Plastics-One, Roanoke, VA, USA) loaded with indian ink into the stereotactic coordinates of the ARC. The loaded cannula was connected via a tube to a 10-µl Hamilton syringe (Hamilton Company) mounted on to an infusion pump (KD Scientific Inc., KDS220). A volume of 0.5µl indian ink was delivered at the speed of 0.25 µl/min in two bilateral injections. The cannula was left for 5 min in the tissue after the injection to minimize back-diffusion and then slowly withdrawn.

Brains were taken out, fixed in 4% PFA and sectioned on a freezing microtome to analyse correct targeting of the ARC. Stereotactic co-ordinates were adjusted to -1.3 mm AP, ± 0.2 mm ML to bregma, and 6.4 mm DV relatively to the skull.

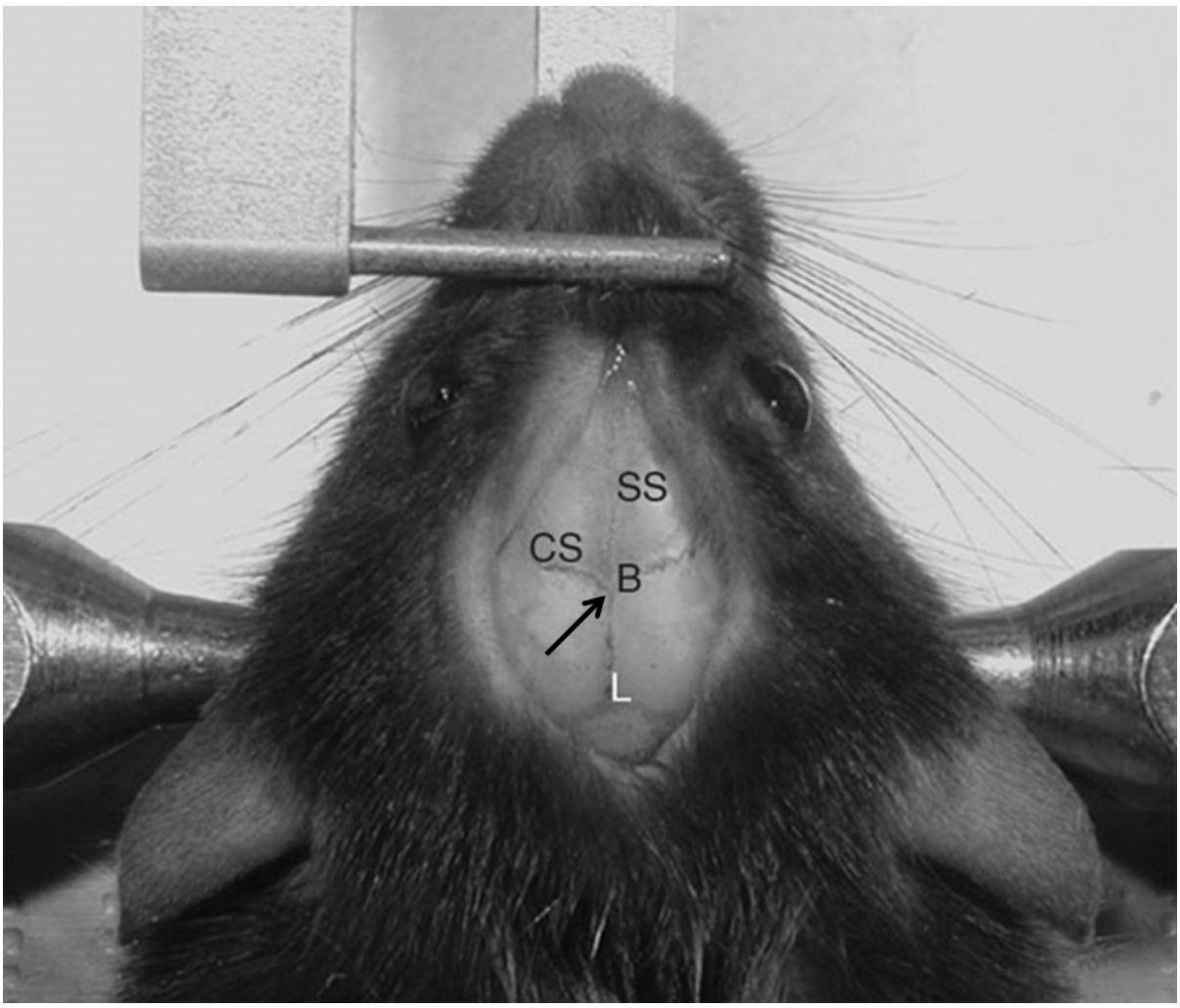


Figure 2.1 - Exposed mouse skull showing the main landmarks. Sagitural suture (SS) and coronal suture of the skull intersection forming bregma (B). Lambda is located at the end of the sagittal suture. The skull is immobilized with aural studs and tooth bar. SS=sagittal sutures B=Bregma, CS= coronal sutures. [424]

2.5.3 Stereotactic surgery

Male mice homozygote for LKB1$^{F/F}$ on a C57Bl/6 background were used for AAV injection into the ARC. Animals were injected with Buprenorphine (0.1mg/kg) prior to surgery to provide analgesia. Animals were placed in an induction chamber and anesthetized with 2%-3% of isoflurane.

Once mice were in deep anaesthesia (confirmed by the absence of a pedal withdrawal reflex to a toe pinch), they were mounted onto the stereotactic frame (KOPF, US) as described above. Ophthalmic lubricant was applied to the anesthetized rodent and a standard heating pad was used during the surgery to provide warmth. Anaesthesia was provided in the form of isoflurane via a gas anaesthesia mask (KOPF, US) mounted on the stereotactic frame.

The bilateral injection and positioning of the skull was performed as described above. This time animals were injected with 0.5µl of AAV2-mIP2-EGFP-T2A-*iCre* or control virus AAV2-mIP2-EGFP-T2A was delivered at the speed of 0.25 µl/min in two bilateral injections. The cannula was left for 5 min in the tissue after the injection to minimize back-diffusion and then slowly withdrawn. The incision in the scalp was closed by interrupted sutures and preheated 0.5ml 0.9% saline was given to prevent dehydration. Animals were allowed to recover in a heated chamber.

2.5.4 Transcardiac perfusion

2.5.4.1 Apparatus and anaesthesia

Animals were killed by an overdose of anaesthesia: Ketamine/Xylazine with 120 mg/kg body weight of ketamine and 24 mg/kg body weight Xylazine in a vehicle containing 0.9% sodium chloride. Syringes (50ml) were either filled with PB (0.1 M phosphate buffer pH 7.2) or 4% paraformaldehyde (PFA) fixative and connected to a blunt perfusion needle.

2.5.4.2 Perfusion surgery

After animal reached deep anaesthesia determined by the toe pinch-response method a lateral incision through the integument and abdominal wall was performed followed by careful separation of the liver from the diaphragm. A subsequent incision in the diaphragm was performed to expose the pleural cavity. The rib cage was cut up to the collarbone as well as on the contralateral side. With a haemostat the sternum was clamped and folded back and tissue connecting the heart was trimmed. Laceration of the right atrial-chamber was followed by penetration of the left ventricle with the blunt perfusion needle and clamped with a haemostat. Animals were then perfused with 30 ml of PB at a slow steady pressure followed by 4% of PFA. Clearing of the liver was used as an indicator of efficient perfusion. Animals were subsequently decapitated and the brain carefully removed from the skull.

2.5.4.3 Post fixation and storage of brains

Brains were further fixed for 1hr or O/N at 4°C in 4% PFA. After post-fixation, brains were washed in PB and cryoprotected in 40% sucrose. Unless used directly, brains were stored in PB with 0.025% sodium azide (SigmaAldrich) and kept at 4°C. Brains were frozen in isopentane cooled down by liquid nitrogen and sliced with 30-µm coronal sections on a freezing sledge microtome. For subsequent immunohistochemistry brain sections were collected in PB and stored at 4°C.

2.6 Statistics

Statistical significance was assessed using Student's t-test with Bonferroni correction, or two way ANOVA, as indicated, using GraphPad Prism 6.0. P-values < 0.05 were considered significant. Values are presented as means ± S.E.M.

Chapter 3 Leptin's role in pancreatic β-cells

Abstract

Background and aims: The highly controlled regulation of pancreatic hormone secretion is vital to maintain functional glucose homeostasis. Defects in the regulation of glucose levels are involved in several metabolic diseases, including type 2 diabetes. Leptin is an adipose tissue-derived satiety hormone with important roles in the maintenance of body weight but has also a role in glucose homeostasis. For instance, leptin administration in *ob/ob* mice reduces their high circulating insulin and glucose levels. Leptin mediates its effects by interaction with leptin receptors (LepRb), which are highly expressed in the hypothalamus and other brain centres, and at lower levels in the periphery.

Previous studies of leptin's effect on glucose homeostasis have yielded inconclusive results *in vitro* as well *in vivo*. *In vivo* studies investigating leptin receptor signalling in pancreatic-β have used promiscuous *Cre* deleter strains, deleting LepRb both in β-cells and in brain areas involved in energy homeostasis.

Methods: Mice were deleted for the LepRb selectively in β-cells with an Ins1*Cre* transgenic line and mice bearing *flox'd* alleles of exon 17 required for leptin receptor signalling. Body weight was recorded and intraperitoneal glucose and insulin tolerance tests were carried out in these mice. Insulin secretion was measured both *in vivo* and *in vitro* using ELISA and homogeneous time-resolved fluorescence- (HTRF) based measurements. Intracellular Ca^{2+} dynamics were assessed by confocal microscopy of the intracellular trapped fluorescent probe Fluo-2 in intact islets. Fluorescence-activated cell sorting (FACS)-purified β, α and δ cells were used to investigate LepRb mRNA expression with quantitative RT-PCR.

Results: LepRb mRNA levels were at or below the level of detection in isolated islets and purified cells from wild-type adult mouse, and leptin signalling to Stat3 phosphorylation was undetectable. Whilst male mice deleted for leptin receptors in β cells exhibited no abnormalities in glucose tolerance up to 16 weeks of age, females transiently displayed improved glucose tolerance at 8 weeks (11.2±3.2% decrease in area under curve; $p<0.05$), and improved (39.0±13.0%, $P<0.05$) glucose-stimulated insulin secretion *in vitro.*

Conclusions/Interpretation: The use here of a highly selective *Cre* recombinase indicates that leptin signalling plays a relatively minor, age- and sex-dependent role in the control of β-cell function in the mouse.

3.1 Introduction

The leptin receptor has previously been deleted *in vivo* in murine pancreatic β cells and in a specific population of neurons in the hypothalamus (RIP2*Cre* neurons) by Covey *et al*., using a *Cre* deleter strain driven by the RIP2-driver. These animals developed obesity, fasting hyperinsulinaemia, impaired glucose-stimulated insulin release and glucose intolerance [192]. On the other hand, Morioka *et al.* disrupted the leptin receptor in all pancreatic cells using a *Pdx1* promoter-driven *Cre* and these mice, in contrast, exhibited normal body weight, improved glucose tolerance and elevated plasma insulin. However, when challenged with high fat diet, Pdx1*Cre*:LepRb^{KO} mice became glucose intolerant [191].

The conflicting results between these two rather similar studies are thus likely to be the result of using different *Cre* lines. In neither case was the leptin receptor deleted exclusively in pancreatic β cells. In RIP2*Cre* mice Cre recombinase is expressed in β-cells as well several brain regions particularly in areas of hypothalamus involved in feeding behaviour and glucose homeostasis.

The Pdx1*Cre* mice used by Morioka *et al.* express *Cre* recombinase in adult β-cells and also glucagon-secreting pancreatic α cells as well as intestinal glucagon-like peptide 1- (GLP-1) secreting L-cells and several other cell types with roles in glucose homeostasis. The Pdx1*Cre* line also deletes LepRb in neuronal populations located in hypothalamic neurons responsive to leptin [193].

To investigate leptin receptors function is in pancreatic β-cell, the use of *Cre*-expressing mice that express recombinase in a strictly β-cell specific manner is essential. Therefore, in this study we have selectively deleted the leptin receptors with a new *Cre*-deleter strain expressing *Cre* strictly in β-cells [400].

3.2 Results

3.2.1 Creation of a β-cell specific LepRb^{KO} mice

To ablate LepRb signalling selectively from pancreatic β cells, animals harbouring alleles in which *Lox*P sites flank exon 17 of the leptin receptor gene [195] were crossed to animals bearing the Ins1*Cre* transgene [400]. Upon *Cre*-expression the LoxP flanked exon 17 is deleted and the terminal exons are disrupted via a frameshift of the coding sequence affecting all the isoforms. Exon 17 includes the BOX1 domain and JAK docking motif and the terminal exons contain the STAT3 phosphorylation motif both required for leptin-induced JAK-STAT signalling. Even though the excision leads to a premature stop codon, the mutated LepRb is expressed but is non-functional [195].

Based on the use of reporter strains [384], the approach is expected to lead to recombination in the majority (>94%) of β-cells, with <2% recombination in other islet cell types [384], in the resulting Ins1*Cre*LepRb^{KO} mice.

To confirm *Cre*-mediated excision of exon 17 (LepRb^{KO}), genomic DNA was isolated from hypothalamus, spleen and islets and PCR performed with three sets of primers to distinguish WT allele with the excised mutant allele as indicated in Figure 3.1A [195]. PCR amplification of DNA from islets isolated from LepRb$^{F/F}$ or Ins1*Cre*LepRb^{KO} animals generated a product of 339bp in all tissues and a band of 208bp where *Cre*-mediated excision of exon 17 had occurred (Figure 3.1B).

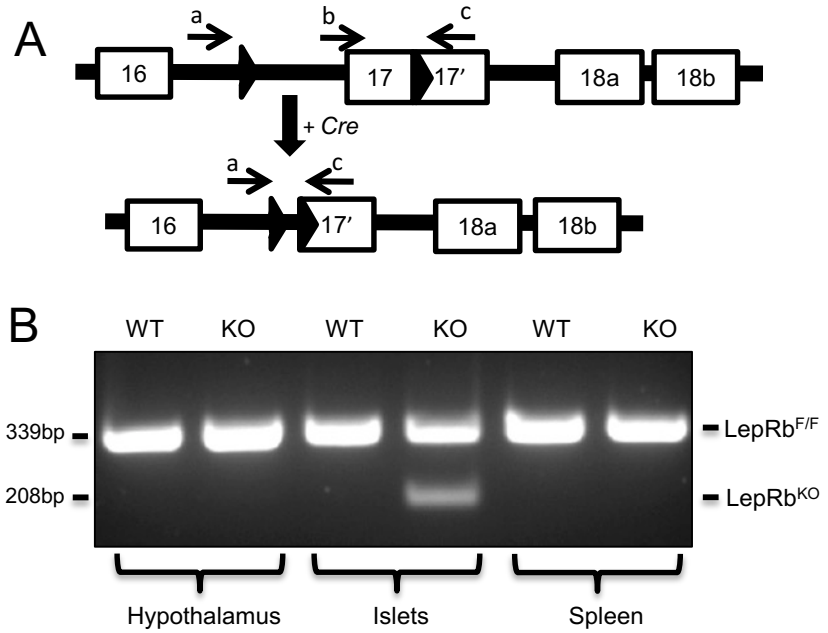


Figure 3.1 - Ins1*Cre* expression results in excision of the *flox*'d region of LepRb alleles selectively in islets. Genomic DNA was harvested from Ins1*Cre*LepRb^{KO} and control LepRb$^{F/F}$ animals and used as a template for PCR with the primers indicated with arrows. White boxes represent exons and black triangles denote loxP sites. Primer (a) binds upstream of the first LoxP site located in intron 16, primer (b) anneals to a sequence exon between the two loxp sites and the communal reversed primer binds behind the last LoxP site located in exon 17. The numbering of the exons in LepR is the same as from [425] **(A)**. Predicted product sizes are 339 bp for the *flox*'d allele (primers b,c) and 208 bp for the excised allele (primers a,c) **(B)**.

As expected none of the control hypothalamus, spleen and islets from LepRb$^{F/F}$ contained the excised band. Whilst attempts were made to confirm deletion by measuring full-length and truncated LepRb mRNA, levels, these were below the level of reliable quantitation both in LepRb$^{F/F}$ and Ins1*Cre*LepRb^{KO} mice, as discussed later.

3.2.2 Similar body weight, fasting blood glucose levels and insulin levels in Ins1*Cre*^{KO} compared to control animals LepRb$^{F/F}$

Bodyweight was followed from the age of weaning (4 weeks) to the age of 16 weeks to investigate if LepRb ablation in β-cell would have an effect on energy homeostasis. Consistent with the absence of recombination and deletion of LepRb in satiety centres in the hypothalamus, loss of LepRb mediated by Ins1*Cre* in the β-cell exerted no effect on body mass in Ins1*Cre*LepRb^{KO} mice (Figure 3.2).

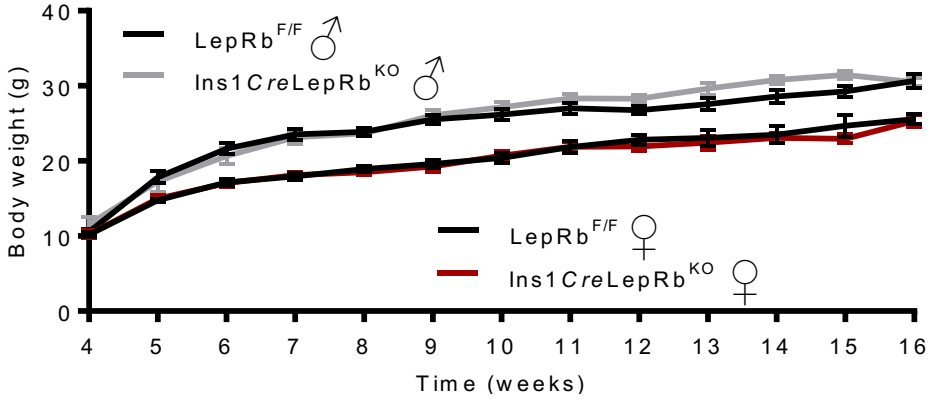


Figure 3.2 - Ins1*Cre*LepRb^{KO} animals displayed a normal progression in bodyweight. Ins1*Cre*LepRb^{KO} males (grey line) and control LepRb$^{F/F}$ (black line) *n*=6-10. Below Ins1*Cre*LepRb^{KO} females (red line) and control LepRb$^{F/F}$ (black line), *n*=6-7. Data are expressed as the average ± SEM. Statistical analysis was performed using two-way ANOVA *P<0.05.

With a reported increase in blood glucose levels in the leptin receptor-deficient mice generated by recombination with RIP2*Cre* [192] but normal blood glucose levels in the Pdx1*Cre* [191], animals was examined for fasting blood glucose levels. Blood glucose concentration was measured after 16hrs over-night fasting. No difference between wild-type and Ins1*Cre*LepRb^{KO} null mice were observed in males or females from the age of 8 weeks to 16 weeks (Figure

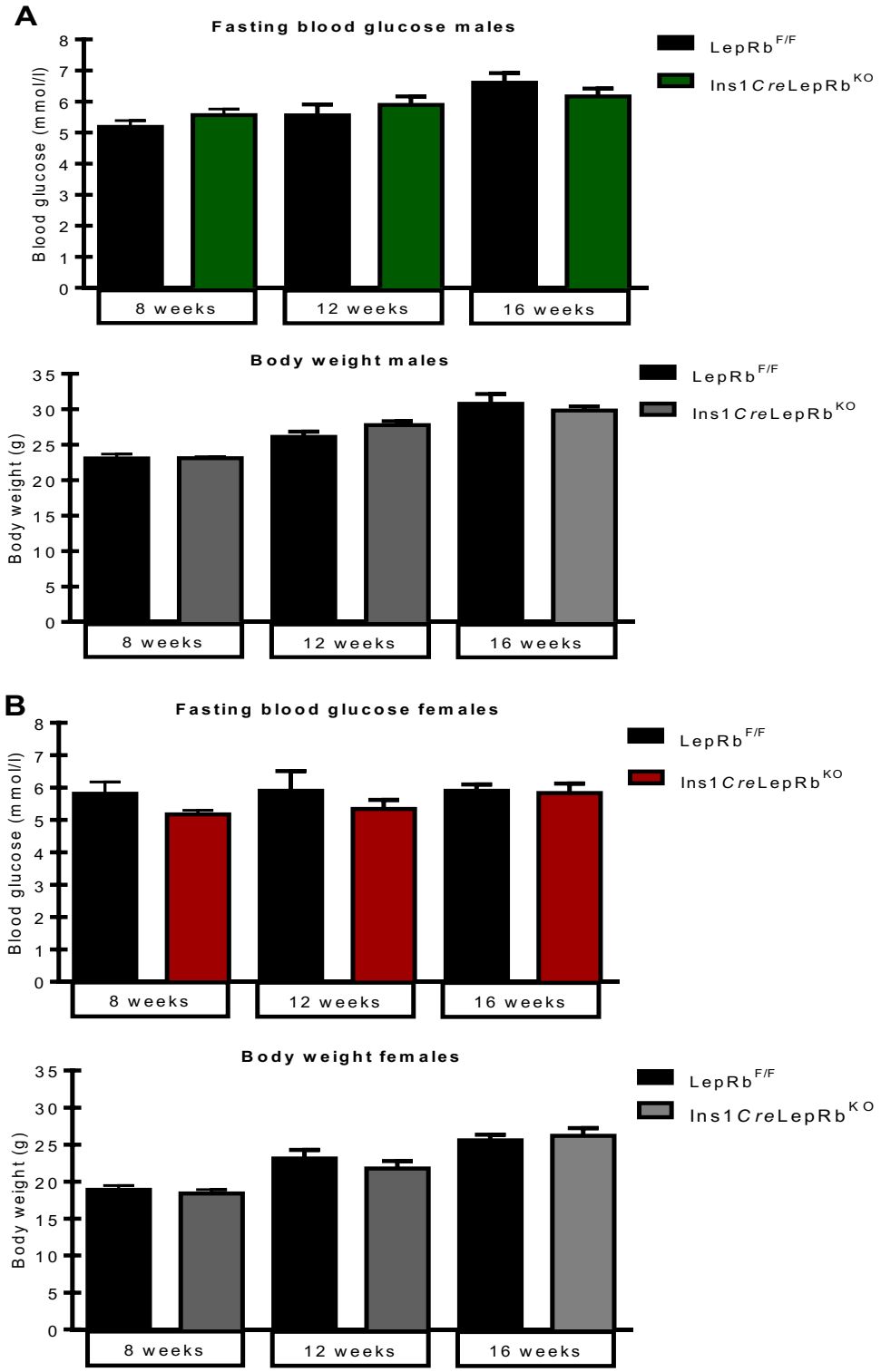


Figure 3.3 - Ins1*Cre*LepRb^{KO} animals demonstrated a normal fasting blood glucose levels. Animals blood glucose levels was measured at the age of 8, 12, 16 weeks. **A**: Ins1*Cre*LepRb^{KO} males (green bar) and control LepRb$^{F/F}$ (black bar) and the graphs below the corresponding weights. 8-week old males: Ins1*Cre*LepRb^{KO}, *n*=8, LepRb$^{F/F}$, *n*=7, 12-week old males Ins1*Cre*LepRb^{KO}, *n*=10, LepRb$^{F/F}$, *n*=10 and 16-week old males: Ins1*Cre*LepRb^{KO}, *n*=13, LepRb$^{F/F}$, *n*=10. **(B)**: Ins1*Cre*LepRb^{KO} females (red bar) and control LepRb$^{F/F}$ (black bar), below their corresponding weights. 8-week old females: Ins1*Cre*LepRb^{KO}, *n*=13, LepRb$^{F/F}$, *n*=9. 12-week old females: Ins1*Cre*LepRb^{KO}, *n*=8, LepRb$^{F/F}$, *n*=5, 16-week old females: Ins1*Cre*LepRb^{KO}, *n*=15, LepRb$^{F/F,}$ *n*=17.
Data are expressed as the average ± SEM. Statistical analysis was performed by student t-test, *P<0.05.

Leptin has been reported in several studies to directly affect insulin secretion from isolated islets (see Chapter, 1, Section 1.5.4.1) suggesting this is achieved via LepRb signalling in islet cells. Secondly, the mice harbouring the RIP2*Cre* or the Pdx1*Cre* mediated deletion of LepRb were both hyperinsulinemic. To determine whether this was an effect due to LepRb deletions in the brain or other cell types or via actions on the LepRb in β-cells, plasma insulin levels were measured in the Ins1*Cre*LepRb^{KO} mice and their littermate controls. In male Ins1*Cre*LepRb^{KO} animals a non-significant (p=0.58) decrease in insulin plasma levels was observed (Figure 3.4). No apparent difference in insulinaemia was detected between genotypes in female animals (Figure 3.4).

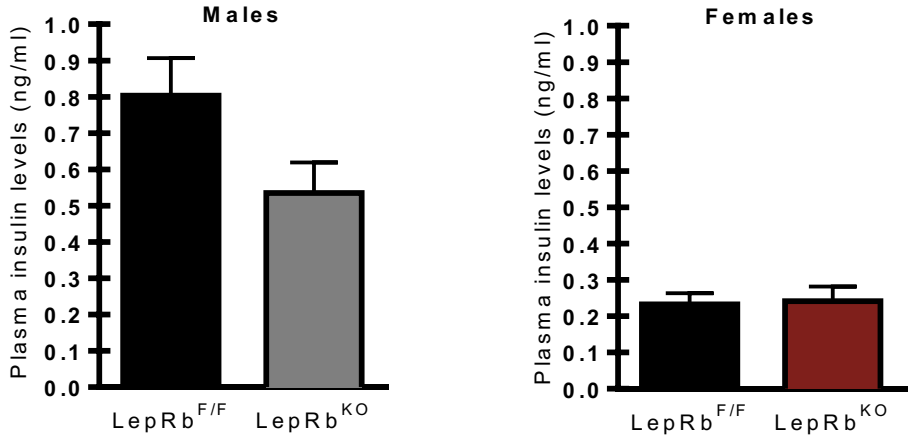


Figure 3.4 - Normal plasma insulin levels in Ins1*Cre*LepRb^{KO}. Animals plasma levels was measured after a 5hrs fast in male Ins1*Cre*LepRb^{KO} (grey bar), n=10 and littermate control LepRb$^{F/F}$ (black bar), n=7, and in females Ins1*Cre*LepRb^{KO} (red bar), n=9, littermate control LepRb$^{F/F}$ (black bar), n=7. Data are expressed as the average ± SEM. Statistical analysis was performed by student t-test, *P<0.05.

3.2.3 No difference in β to α cell ratio after LepR deletion from β cells

Both of the previously described leptin receptor-deficient mice, generated by recombination with RIP2*Cre*- [192] or Pdx1*Cre*- [191] delete strains, displayed an abnormal β cell area, suggesting LepRb signalling is involved in controlling β-cell mass. Therefore, we investigated β-cell to α-cell ratio in paraffin embedded pancreatic slices from Ins1*Cre*LepRb^{KO} and littermate control. To examine the β to α cell ratio pancreas was stained with anti-insulin and anti-glucagon antisera and images were captured with an inverted widefield microscope fitted with a Hamamatsu Flash 4.0 Camera using a Plan-Apochromat 206/0.8 M27 air objective with Colibri.2 LED illumination. Fluorescent quantification was achieved using Image J and the β to α cell ratio were calculated using an in-house macro for comparative analysis between sample groups, no changes in this parameter were apparent (Figure 3.5).

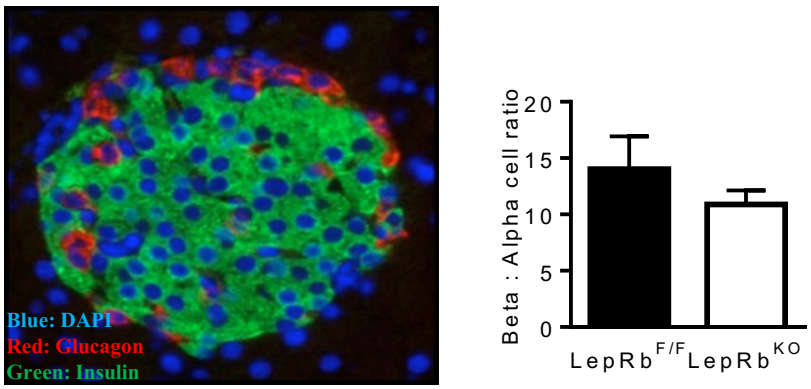


Figure 3.5 - Normal β to α cell ratio in Ins1*Cre*LepRb^{KO}. Staining of paraffin embedded pancreatic tissue. **(A)** Representative picture of staining of an islet from a WT mouse (green represents insulin, red glucagon and blue dapi). **(B)** No difference in beta to alpha cell ratio was observed between Ins1*Cre*LepRb^{KO} and control LepRb$^{F/F}$, *n*=3 animals per genotype. Data are expressed as average ± SEM. Statistical analysis was performed by student t-test, *P<0.05.

3.2.4 Gender and age-dependent effects on glucose tolerance of LepRb deletion from β-cells

To examine in more detail whether disruption of leptin signalling in β-cells affects glucose metabolism, animals aged 8, 12 and 16 weeks were subjected to intraperitoneal glucose tolerance tests (IPGTT; males, figure 3.6A, females, (figure 3.6B). No difference between KOs and controls was observed in male animals at any of the ages examined. At 8 weeks, female Ins1*Cre*LepRb^{KO} null mice showed transiently improved glucose tolerance (11.2±3.2% decrease in area under curve; $p<0.05$), which was reversed by the age of 12 weeks and 16 weeks (Figure 3.6B).

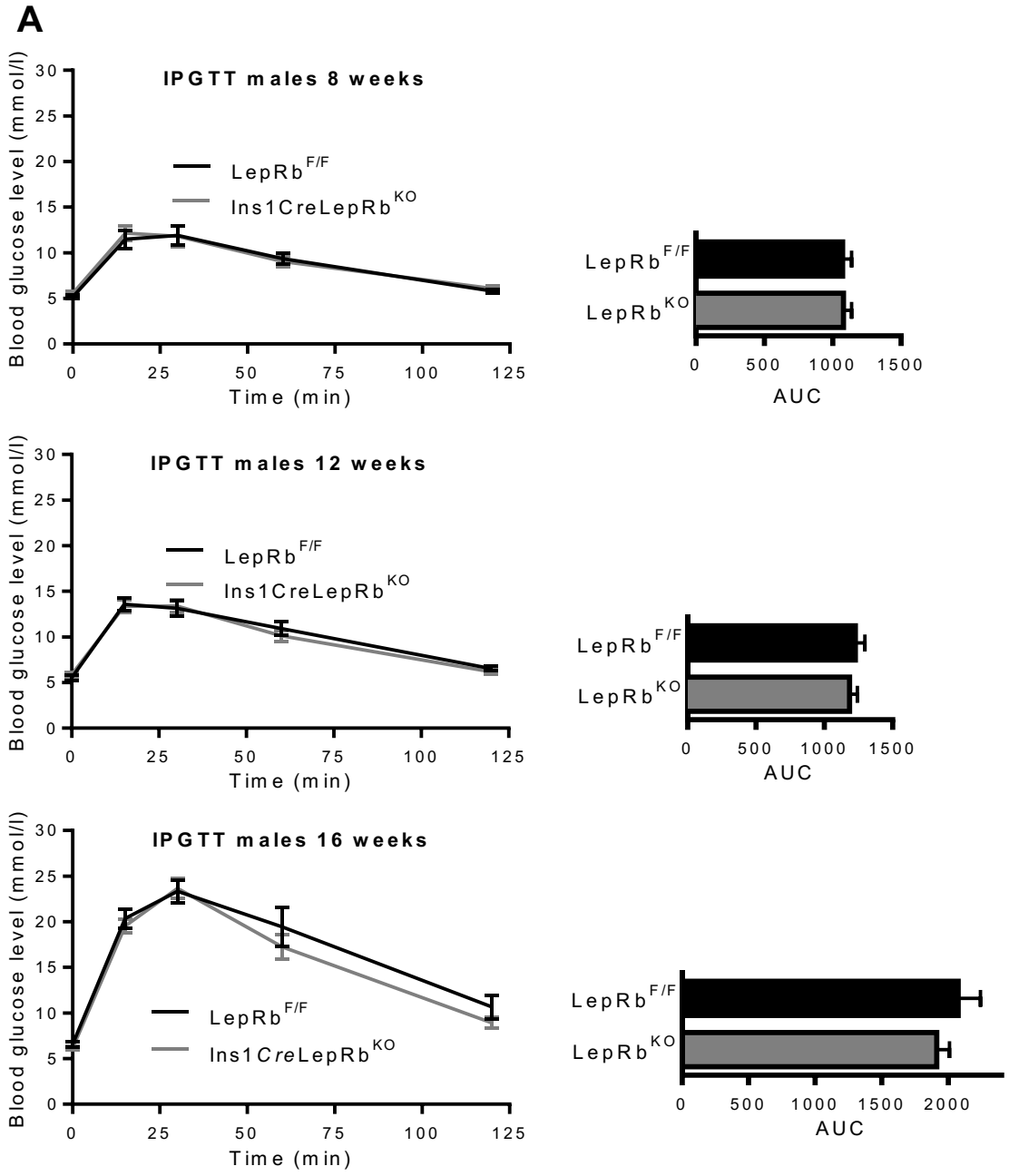


Figure 3.6 A - Ins1*Cre*LepRb^{KO} males display no change glucose tolerance at the age of 8 to 16 weeks. Ins1*Cre*LepRb^{KO} animals were compared to littermate controls LepRb$^{F/F}$ at equivalent age. Mice were fasted overnight and challenged with glucose (1g/kg body weight). Blood glucose concentration following IPGTT in Ins1*Cre*LepRb^{KO} (grey line) and LepRb$^{F/F}$ littermate controls (black line). **Top**: IPGTT in 8-week old males: Ins1*Cre*LepRb^{KO}, *n*=8, LepRb$^{F/F}$, *n*=7. **Middle:**12-week old males: Ins1*Cre*LepRb^{KO}, *n*=10, LepRb$^{F/F}$, *n*=10. **Bottom:** 16-week old males: Ins1*Cre*LepRb^{KO}, *n*=13, LepRb$^{F/F}$, *n*=10. For all panels, the area under curve (AUC) is displayed on the right. Data are expressed as the average ± SEM. Statistical analysis was performed by 2-way ANOVA or student t-test, *P<0.05.

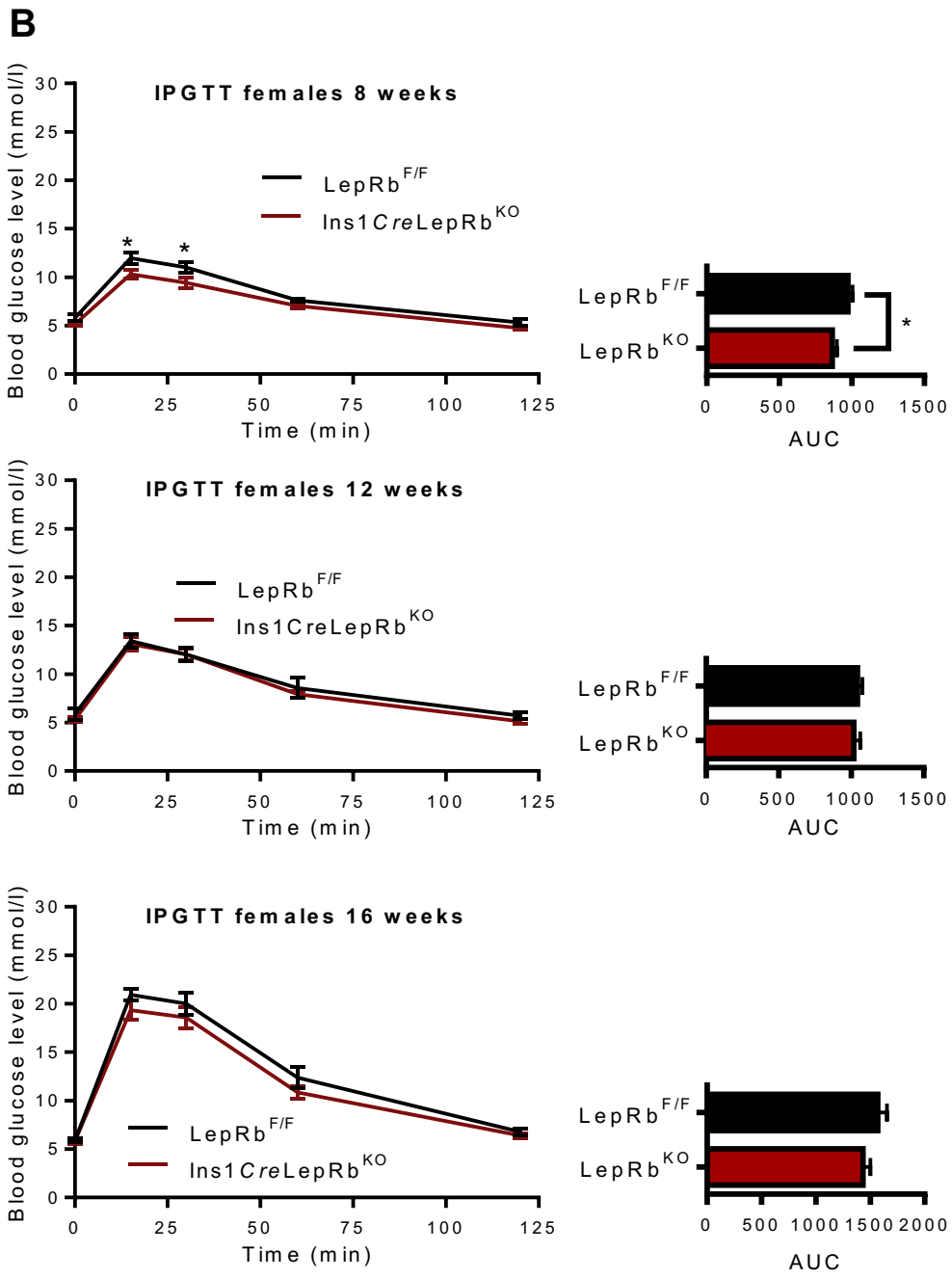


Figure 3.6 B - Ins1*Cre*LepRb^{KO} females display improved glucose tolerance at the age of 8 weeks. Animals were fasted overnight and blood glucose levels measured after challenge with 1g/kg in 8-week old females: Ins1*Cre*LepRb^{KO} (red line), *n*=13, LepRb$^{F/F}$ (black line), *n*=9 (top). 12-week old females: Ins1*Cre*LepRb^{KO}, *n*=8, LepRb$^{F/F}$, *n*=5, 16-week old females: Ins1*Cre*LepRb^{KO}, *n*=15, LepRb$^{F/F,}$ *n*=17(bottom). For all panels, the area under curve (AUC) is inset. Data are expressed as the average ± SEM. Statistical analysis was performed by 2-way ANOVA or student t-test, *P<0.05.

3.2.5 Insulin sensitivity and glucose stimulated insulin secretion is normal

Both male and female RIP2*Cre*-mediated LepRb-ablated mice had impaired insulin sensitivity whereas in the Pdx1*Cre*-mediated LepRb ablation animals had no insulinemic phenotype. Therefore, following a 5hr fast, Ins1*Cre*LepR^{KO} mice were examined for whole-body insulin sensitivity (Figure 3.7). No difference was observed between Ins1*Cre*LepRb^{KO} and wildtype littermates LepRb$^{F/F}$.

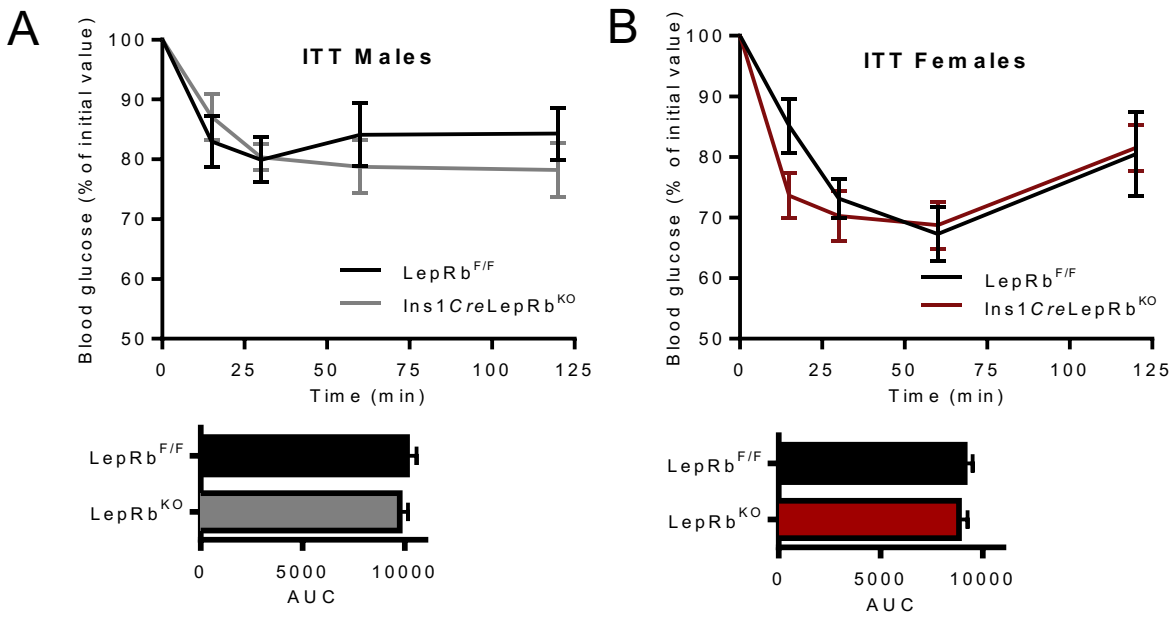


Figure 3.7 - Ins1*Cre*LepRb^{KO} animals showed no difference in insulin sensitivity. **(A)** Blood glucose concentration following ITT in male Ins1*Cre*LepRb^{KO}, *n*=10 (grey line) and LepRb$^{F/F}$, *n*=9 littermate controls (black line). Female animals Ins1*Cre*LepRb^{KO}, *n*=10 (red line) and littermate LepRb$^{F/F}$, *n*=9 (black line). For all panels, the area under curve (AUC) is inset. Data are expressed as the average ± SEM. Mice were aged 15-16 weeks.

Since RIP2*Cre* animals with ablation of LepRb display an impaired insulin response to glucose [192], whereas the Pdx1*Cre* animals had enhanced insulin secretion in the acute phase [191], therefore, we examined these mice for glucose stimulated insulin secretion GSIS *in vivo*. Animals were challenged with 2g glucose/kg mouse weight followed by tail vein blood collection at time points 0, 15 and 30 min for plasma insulin measurements. No difference was observed between the mutant and the wild type animals in insulin response to the glucose challenge (Figure 3.8).

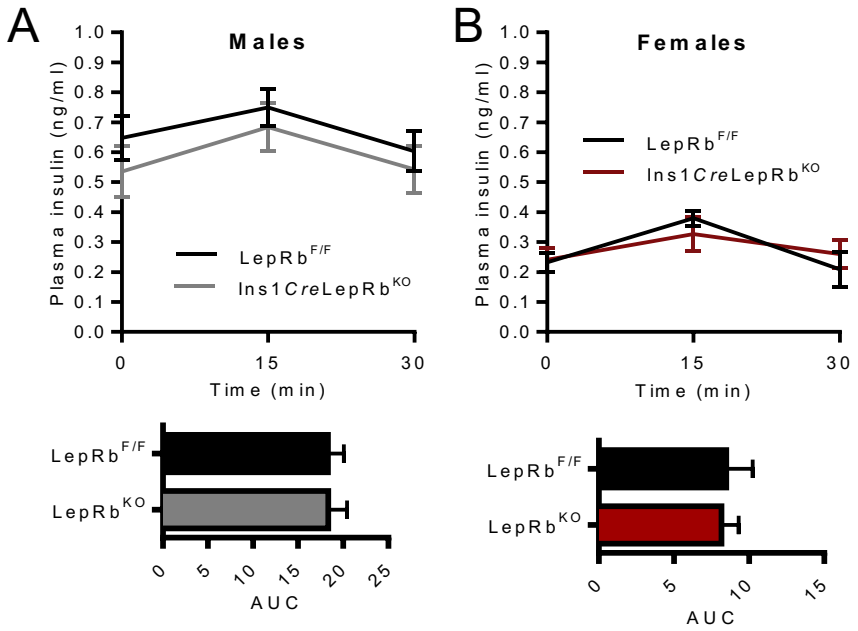


Figure 3.8 - Ins1*Cre*LepRb^{KO} animals displayed no difference in GSIS. (A) Plasma insulin levels following IP injection of glucose in male Ins1*Cre*LepRb^{KO}, *n*=10 (grey line) and LepRb$^{F/F}$, *n*= 7 littermate controls (black line). **(B)** Female animals Ins1*Cre*LepRb^{KO}, *n*=10 (red line) and littermate LepRb$^{F/F}$, *n*=6 (black line). Inset below: corresponding area under curve (AUC). Data are expressed as the average ± SEM.

3.2.6 Enhanced insulin secretion in isolated islets from female KO mice

Several research groups have reported leptin having an effect on insulin secretion in isolated islets at glucose concentrations stimulating insulin secretion (GSIS), either by inhibition [174-176, 179, 182, 190] or in contrast by stimulating insulin secretion [181]. However, there are also several reports who have observed no effect on insulin secretion at glucose stimulating insulin secretion concentrations [177, 178, 180-185, 187, 189].

Furthermore, Morioka and colleagues demonstrated enhanced insulin secretion in isolated islets from Pdx1*Cre* LepRb KO animals under GSIS concentrations [191]. In contrast, the RIP2*Cre*LepRb^{KO} mice had a 47% decrease in secretion of insulin in comparison to WT controls. It was also observed that RIP2*Cre*LepRb^{KO} islets secreted 1.4 fold more insulin at basal glucose concentration [192].

To determine if the leptin receptor in pancreatic β-cells mediates an effect on insulin release, insulin secretion from isolated islets from Ins1*Cre*LepRb^{KO} was compared to wild type. Islets was isolated and incubated for 30 min at either low glucose (LG, 3mM glucose), high glucose (HG, 17mM glucose) or 30mM KCl followed by the collection of secreted insulin and total insulin and measurement with homogeneous time-resolved fluorescence-based HTRF-kit (Figure 3.9A,B).

We observed that isolated islets from female Ins1*Cre*LepRb^{KO} animals had an enhanced insulin secretion at high glucose levels (39.0±13.0%, P<0.05), (figure 3.9A) compared to those from RIP2*Cre*LepRb^{KO} animals. However, no difference in insulin secretion was observed in islets from male animals (Figure 3.9B). Despite the difference in insulin secretion at high glucose levels, isolated islets were equally responsive in insulin release after addition of KCl in females and the same result was observed in males. No difference between Ins1*Cre*LepRb^{KO} and control islets was observed at low glucose concentrations in either gender (Figure 3.9A,B).

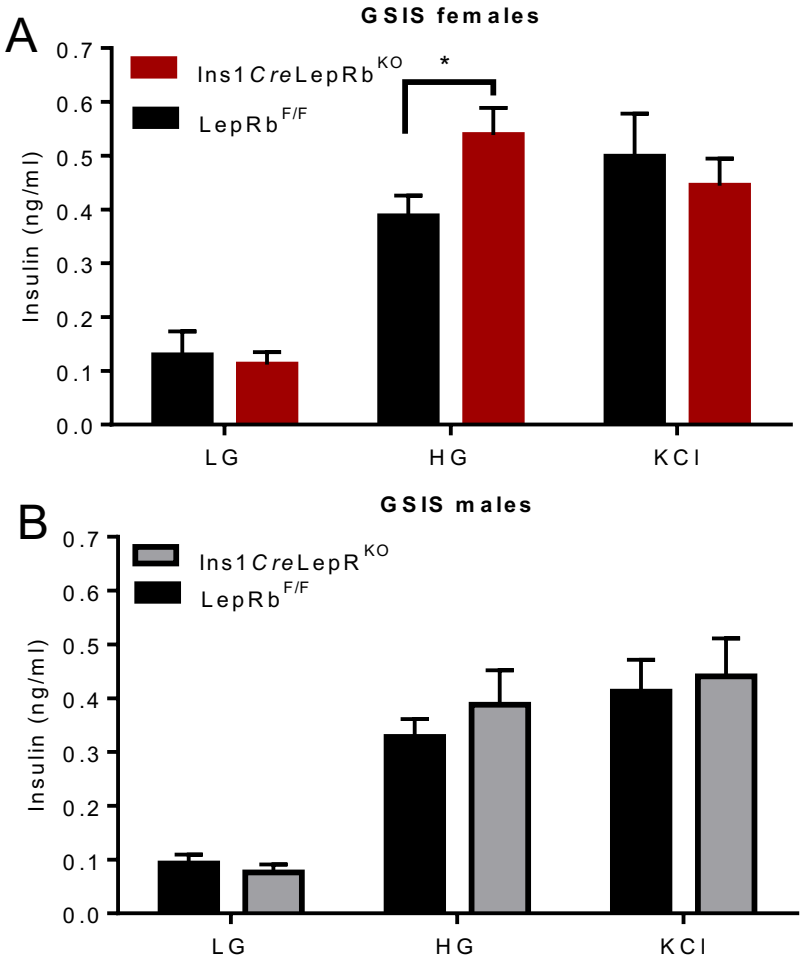


Figure 3.9 - Enhanced glucose-stimulated insulin secretion in Ins1*Cre*LepR^{KO} females. Insulin secretion as assessed from isolated islets in response to glucose (LG=3 mM, HG = 17 mM) and KCl (30 mM) in 30-week old female **(A)** and male **(B)** mice**.** *n*=3 separate experiments involving 2-3 mice per genotype/experiment. Statistical analysis was performed using Student's t-test. *P<0.05.

3.2.7 Deletion of leptin receptors has no effect on glucose-induced increases in intracellular free Ca^{2+}

Glucose dependent insulin secretion involves glucose uptake that induces elevations of intracellular ATP/ADP ratio, followed by membrane depolarization, Ca^{2+} entry through voltage-dependent Ca^{2+}-channels to increase intracellular (cytosolic) free calcium($[Ca^{2+}]i$) and lastly secretion of insulin by exocytosis of insulin containing granules (See Chapter 1, Section 1.2.1.3).

Deletion of LepRb in β-cells using the RIP2*Cre* promoter resulted in impaired glucose dependent Ca^{2+} signalling within the islets [426]. The RIP2*Cre* animals had impaired intracellular Ca^{2+} oscillations and a lack of synchrony in 57% of all islets examined whereas all $LepRb^{F/F}$ control animals had normal oscillations. The same defects was observed in neonatal islets suggesting this phenotype is not due to the obesity that these animals also suffer from [426]. However, these authors also observed a defect in control animals harbouring only the RIP2*Cre* transgene. The Ins1*Cre* on the other hand has normal body weight gain, glycaemic levels and glucose tolerance [400]

Contrary to the above findings, deletion of LepRb with the Pdx1*Cre* promoter resulted in a significantly enhanced glucose stimulated Ca^{2+} flux. These authors also observed in control animals that treatment with leptin suppressed the Ca^{2+} flux, an effect that was missing in islets lacking the LepRb [191].

Considering that insulin secretion in response to KCl is similar in the Ins1*Cre*$LepRb^{KO}$ animals as to the $LepRb^{F/F}$ controls confirms that this machinery is intact in the β-cells (figure 3.10A*).* To examine if the more selective Ins1*Cre* mediated deletion of LepRb is affecting the Ca^{2+} flux, changes in intracellular Ca^{2+} were monitored after an elevation of glucose from 3mM to 11 mM (figure 3.10) or depolarization with 30 mM KCl (figure 3.11).

Recordings for around 30 min in islets lacking LepRb showed that islets had normal intracellular Ca^{2+} oscillations and synchrony in comparison to control islets ($LepRb^{F/F}$) (Figure 3.10A) and no difference was observed in the area under curve of Ca^{2+} fluxes (figure 3.10B). Islets deleted for LepRb did display a significantly lower amplitude of $[Ca^{2+}]_{cyt}$ in response to glucose, (Figure 3.10C). However, when hundreds of single cells were individually assessed within each islet to exclude cells not responding to glucose, and those cells displaying large and sustained increases indicative of apoptosis, the significant difference was lost (figure 3.10D).

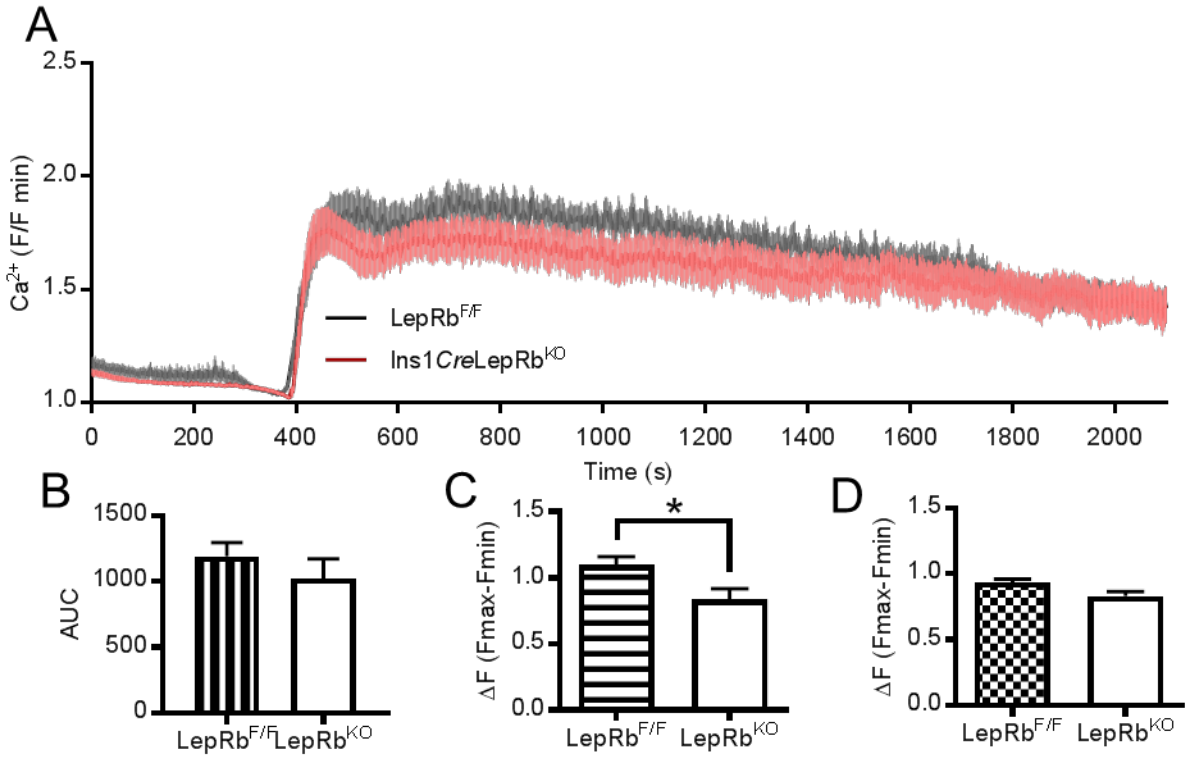


Figure 3.10 - Ins1*Cre*LepRb^{KO} islets display normal Ca^{2+} responses to high glucose. (A) Ca^{2+} recordings from control LepRb$^{F/F}$ islets (black line) and Ins1*Cre*LepRb^{KO} islets (red line) in response to a step-up of glucose concentration from 3mM to 11 mM glucose at 300s. **(B)** Area under the curve (AUC). Amplitudes (ΔF (F_{max}-F_{min})) of the Ca^{2+} rises **(C)**. Amplitudes after removal of glucose non-responsive cells ΔF (F_{max}-F_{min})) of the Ca^{2+} rises **(D)**. Data are from 12-16 islets (3-4 mice) per experiment and statistical analysis was performed using Student's t-test. *P<0.05.

Islets were also investigated for Ca^{2+} rises after depolarization with KCl. LepRb$^{F/F}$ islets were found to have increased Ca^{2+} amplitude in comparison to islets lacking LepRb (Figure 3.11). A tendency for a lower area under curve was observed in Ins1*Cre*LepRb^{KO} islet but not significant (p=0.645) (Figure 3.11 insert). There is a significant difference in the glucose induced Ca^{2+} excursion between LepRb$^{F/F}$ control animals and Ins1*Cre*LepRb^{KO} animals. The glucose induced Ca^{2+} amplitude $\Delta F(F_{min}$-$F_{max})$ was higher in LepRb$^{F/F}$ animals compared to animals deleted for the LepRb (figure 3.11;p=0.02).

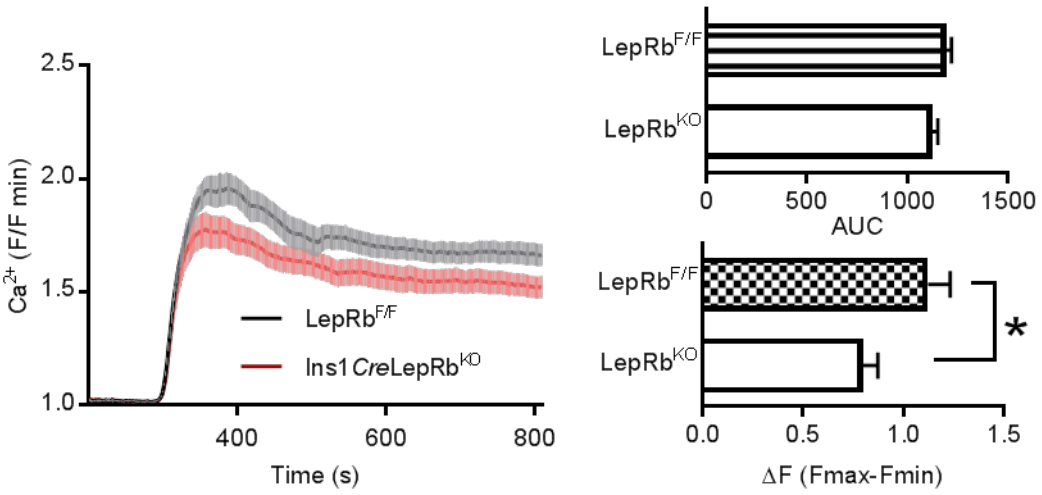


Figure 3.11 - Ins1*Cre*LepRb^{KO} islets display decreased Ca^{2+} responses to KCl. Ca^{2+} recordings from control LepRb$^{F/F}$ islets (black line) and Ins1*Cre*LepRb^{KO} islets (red line) in response to an increase in KCl concentration. **Inset:** area under the curve (AUC) and amplitudes (ΔF (F_{max}-F_{min})) of the Ca^{2+} rises, Data are from 12-16 islets (3-4 mice) per experiment and statistical analysis was performed using Student's t-test. *P<0.05.

Islet Ca^{2+} recordings were analysed for β-cell to β-cell connectivity [423]. No difference in β-cell to β-cell connectivity was observed between LepRb$^{F/F}$ islets and islets deleted for LepRb during the transition of low glucose 3mM to high glucose 11mM, nor during the high glucose phase, suggesting that LepRb is not required for a coordinated β-cell population response to glucose, Figure 3.12 and 3.13.

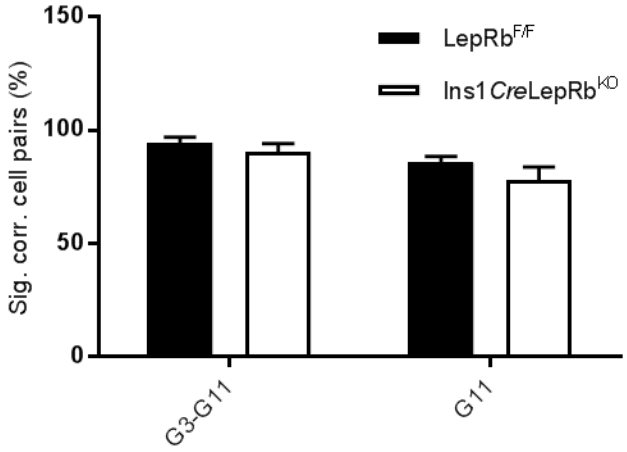


Figure 3.12 - Ins1*Cre*LepRb^{KO} islets had normal β-cell to β-cell connectivity. Connectivity analysis [423] showing the proportion correlated cell pairs during a step change from 3 – 11 mM glucose (*i.e.* the activity onsets), or at steady state in the continued presence of 11 mM glucose. Statistical analysis was performed using Student's t-test.

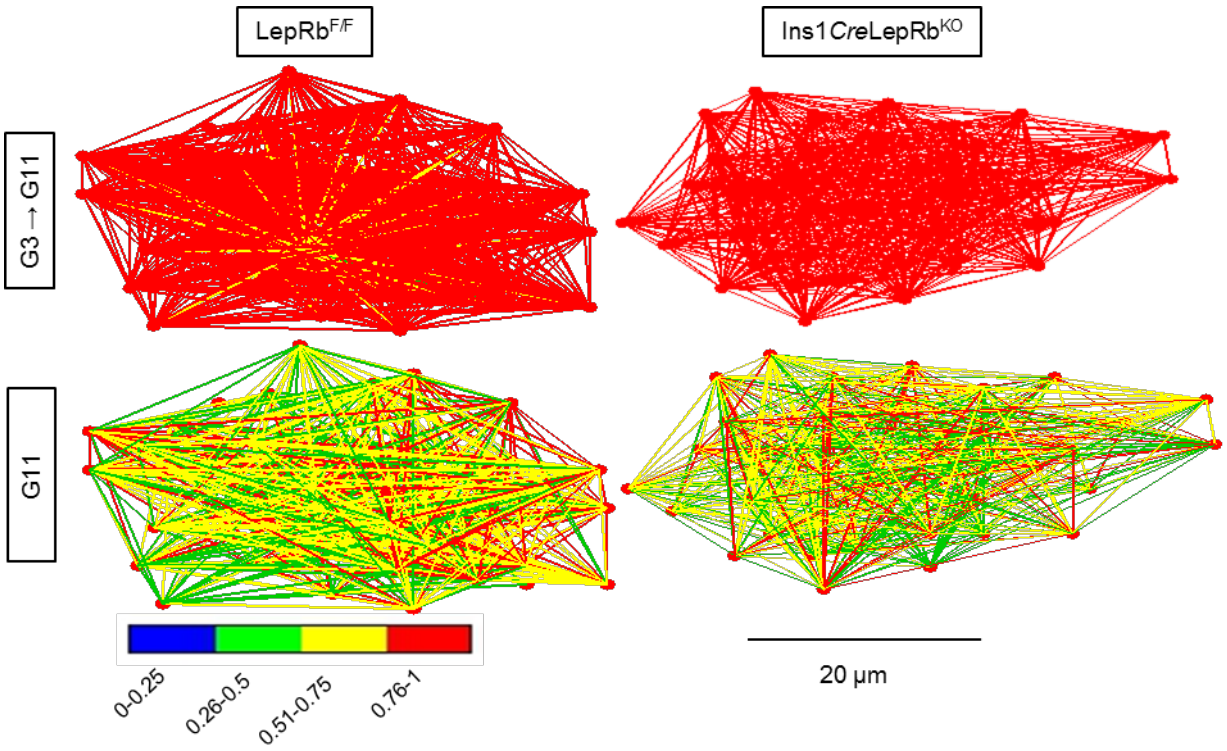


Figure 3.13 - Representative connectivity maps of Ins1*Cre*LepRb^{KO} and LepRb$^{F/F}$. Connectivity analysis [423] showing representative connectivity maps displaying the location (X-Y) in control islets from LepRb$^{F/F}$ animals (left side) and in islets from Ins1*Cre*LepRb$^{F/F}$ (right side). Pseudocolor plots are demonstrating the strength of cell to cell connections, determined by using Pearson R (blue=lowest and red=highest).

3.2.8 LepRb expression is low in isolated mouse islets and with no detectable expression in purified islet cells

To confirm deletion of exon 17 in the Ins1*Cre*LepRb^{KO} islets, full-length and truncated LepRb mRNA levels were examined; however, these were below the level of reliable quantitation both in LepRb$^{F/F}$ and Ins1*Cre*LepRb^{KO} mice. Primers for quantitation of mRNA were designed so the forward primer binds the exon junction of exon 16 and the excised exon 17 and the reversed primer binds to exon 17, creating an amplicon of 89 nt in LepRb$^{F/F}$ and no amplification when exon 17 is excised by *Cre* recombination. The amplification efficiency of the primers was checked with serial dilutions in isolated RNA from hypothalamus, where the expression of LepRb is known to be abundant [199].

To further examine the LepRb expression in islets, qRT-PCR was performed using mRNA prepared from the three isolated cell preparations, β, α and δ-cells, figure 3.14 A-C. Purified β, α and δ-cells were prepared by Dr Alice Adrianssens (University of Cambridge, U.K.) as described in Appendix 2. Briefly, sorting of pancreatic δ-cells was enabled by using islets from transgenic animals harbouring EYFP under the control of the prosomatostatin promoter [427] isolation of pancreatic β- and α-cells were enabled with the use of

animals harbouring the fluorescent protein Venus under the control of the glucagon promoter [286]. Following isolation from the above animals, islets were dispersed and FACS sorted and RNA extracted.

The mRNA isolated from purified β, α and δ-cells, and additionally mRNA from the hypothalamus, were used in qRT-PCR analysis to amplify LepRb. This approached failed to amplify a product from any of the three purified islet cells (Fig. 3.14 A-C). However, the primers efficiently amplified LepRb mRNA from hypothalamus derived mRNA, Figure 3.14D.

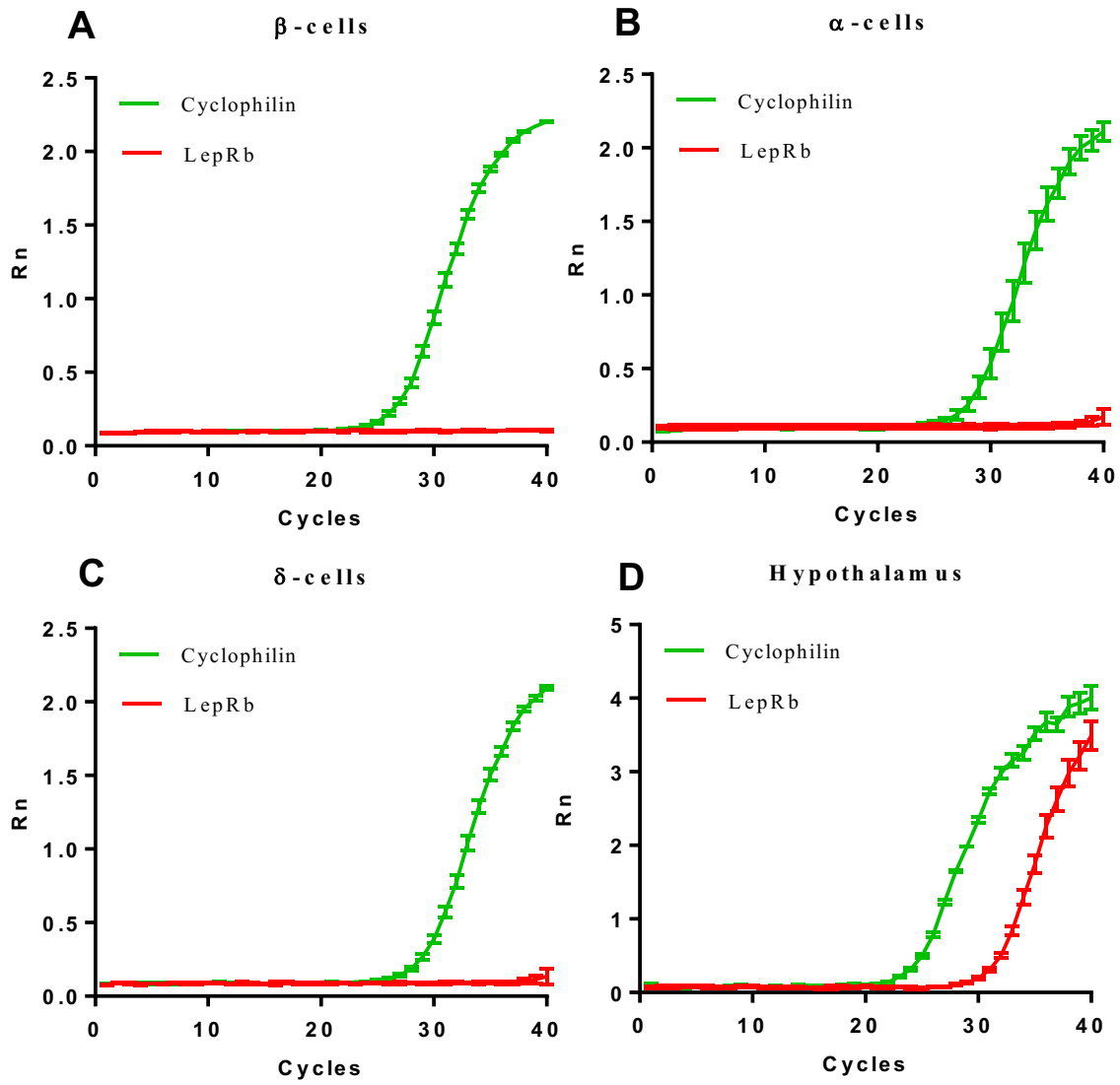


Figure 3.14 - Low levels of LepRb mRNA and receptor signalling from FACS sorted β, α or δ-pancreatic islet cells. Amplification plot of LepRb (red) or Ppia (cyclophilin, green) using mRNA purified from mouse islet **(A)** β-cells **(B)** α or **(C)** δ cells or **(D)** hypothalamus.

The leptin receptor signals through the JAK/STAT pathway and phosphorylation of Y705 on STAT3 is necessary for the receptor to signal via this pathway. To further test the hypothesis that LepRb signalling is

low or undetectable in pancreatic islet cells, we investigated if leptin would induce phosphorylation of STAT3. The Stat3 pathway is in addition to leptin activated by several other ligands such as interleukins and has been demonstrated to signal in islets [428, 429]. Isolated islets from wild type C57BL6 mice were exposed to either leptin, or to a cytokine mixture (IL1-β and TNF-α) as a positive control, and monitored Stat3 phosphorylation on Y705 using immunocytochemistry and laser-scanning confocal imaging.

Whereas the cytokine mixture caused a robust increase in Stat3 phosphorylation as anticipated, no effects were observed upon addition of leptin, even at the highest dose (10 nM) tested (Figure 3.15).

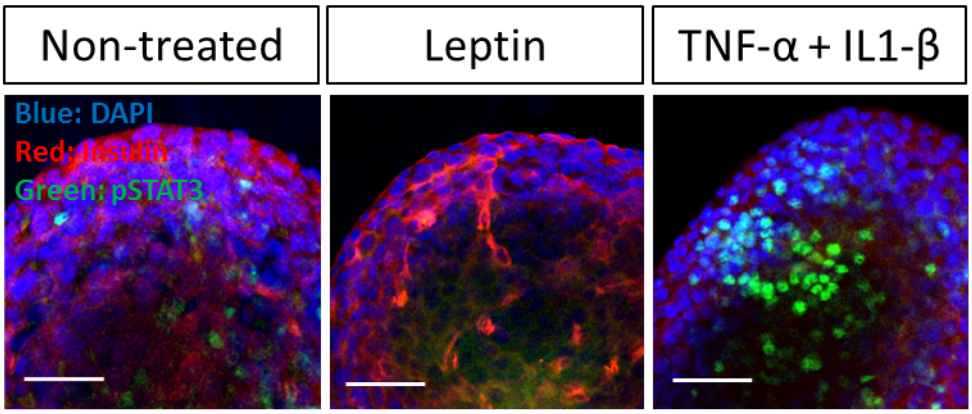


Figure 3.15 - Leptin failed to induce STAT3 phosphorylation in isolated islets. Immunofluorescence staining of pStat3 in whole islets after treatment with either leptin (10 nM), TNF-α and IL1-β, or control non-treated islets. Blue=DAPI, red=insulin, green=pSTAT3.Data are representative of two further experiments. Scale bar 52.5 µm. See the Materials and Methods section for further details.

To further examine the STAT3 phosphorylation, signals from islets were captured with laser scanning widefield imaging (Figure 3.16), using the volocity software and analysed for intensity of the pSTAT3 signals. The cytokine mixture revealed strong Stat3 phosphorylation, with significantly less signalling in leptin treated islets. Interestingly, the non-treated islets revealed a higher signal than the leptin treated (Figure 3.16).

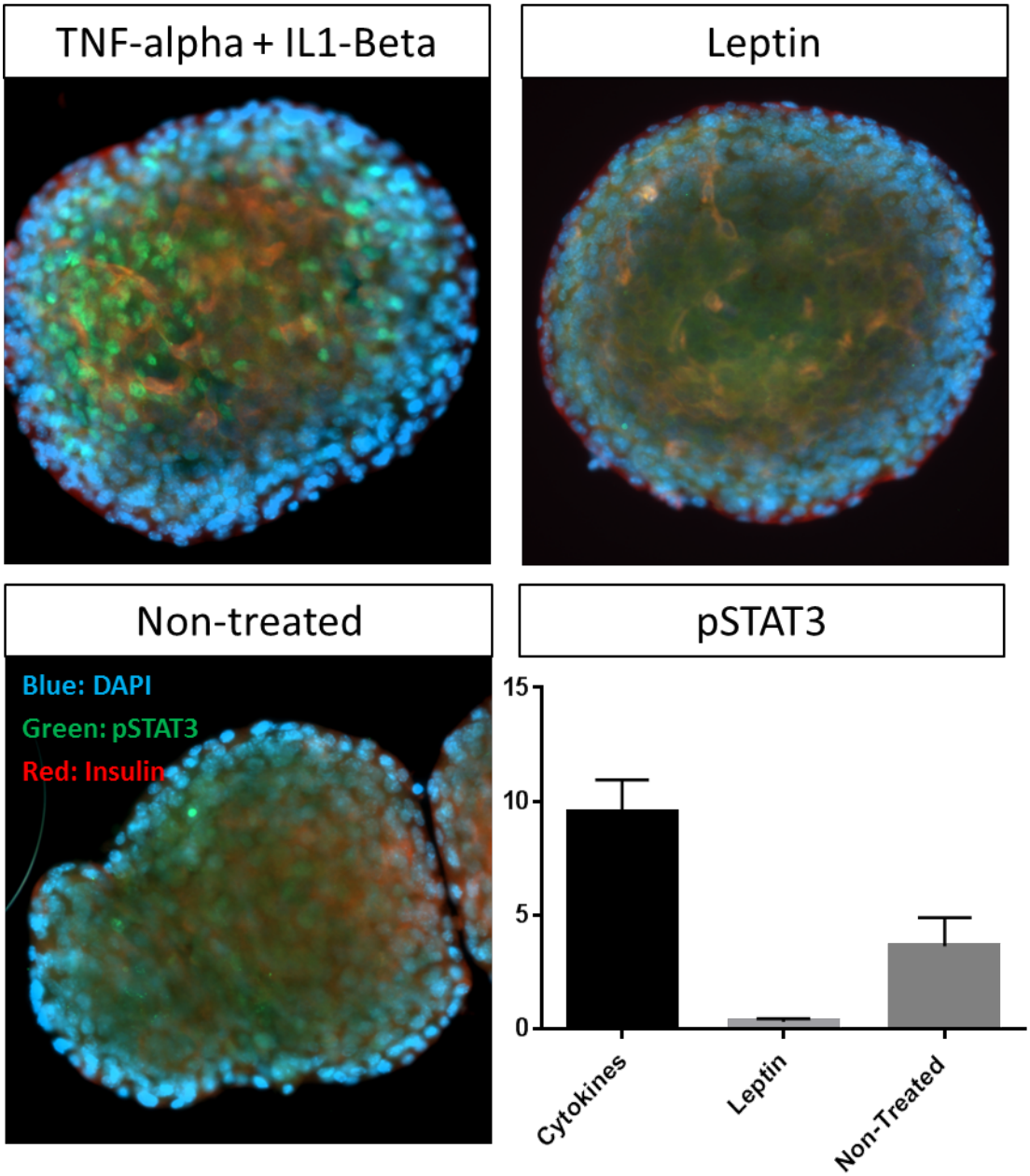


Figure 3.16 - Signal of pSTAT3 is robust in cytokine treated islets but not in leptin treated. Immunofluorescence staining of pSTAT3 in whole islets after treatment with either TNF-α and IL1- β or leptin (10 nM), or control non-treated islets. Blue=DAPI, red=insulin, green=pSTAT3. Graph represents quantification of the signal intensity of pSTAT3 in islets treated with cytokines, leptin and control non-treated. Data are representative of two further experiments.

These findings together suggest that leptin receptor levels are vanishingly low (β, δ) or zero (α) in key islet cell types and limit the actions of the hormone on this tissue.

3.2.9 Confirmation of recombinant leptin ability to induce pSTAT3 in hypothalamus

In an additional experiment, we confirmed that the leptin used in our experiments was functional and able to phosphorylate pSTAT3. Leptin was injected IP into live wild-type mice followed by staining for pSTAT3 in the arcuate nucleus of hypothlamus. Wildtype mice were fasted overnight to then either injected IP with saline (control) or recombinant leptin and sacrificed 40 min after injection with transcardiac perfusion. Brains were removed, sliced and stained for STAT3 phosphorylation using a phosphoSTAT3-specific antibody.

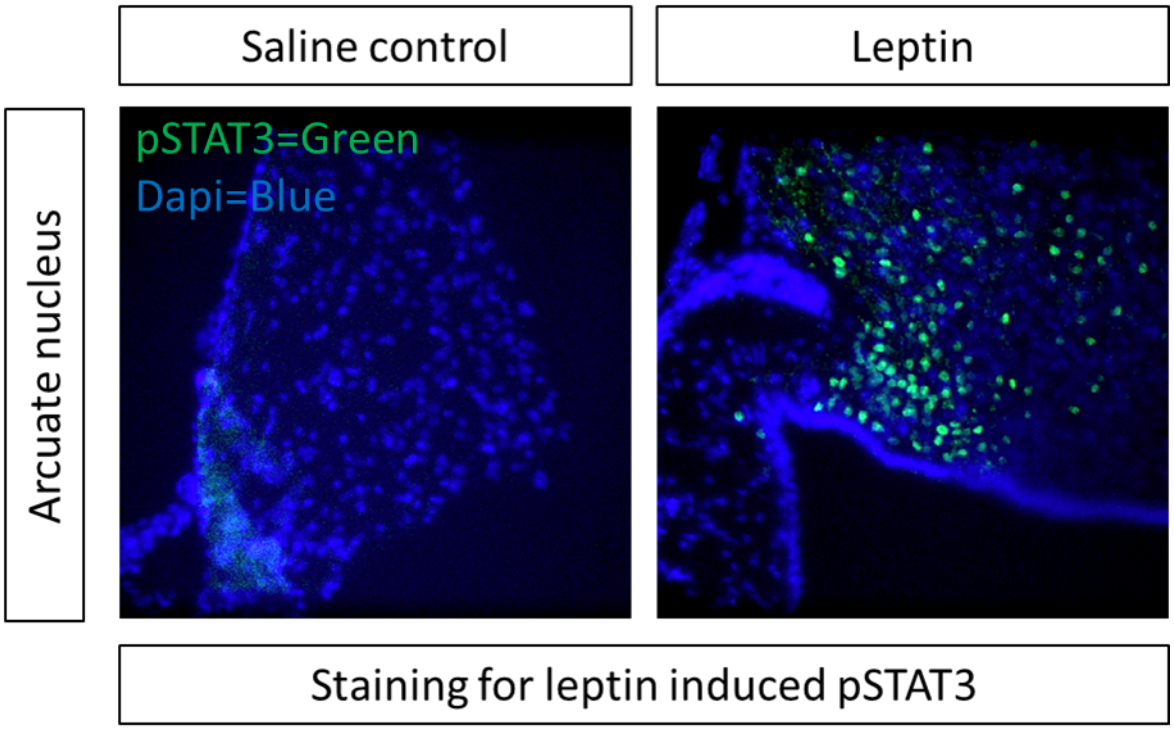


Figure 3.17 - Fasted animals showed STAT3 phosphorylation only upon administration of leptin. Immunofluorescence staining of pStat3 in the arcuate nucleus of hypothalamus from fasted animals injected with control saline **(left)** or recombinant leptin (5mg/kg), **(right)**. Blue=Dapi, green=pSTAT3.

Phosphorylation of STAT3 in the arcuate nucleus of hypothalamus was only observed upon leptin administration, as fasted animals exhibited a near total absence of STAT3 phosphorylation in saline-injected controls, consistent with results from [430], (Figure 3.17). This result confirms that our leptin was functional and able to induce pSTAT3 phosphorylation in animals.

3.3 Discussion

Our study presents the first examination of the effect of a strictly β-cell specific deletion of leptin receptor function using Ins1*Cre*-mediated deletion.

3.3.1 Detection of leptin receptor expression in highly purified β-cell

The study demonstrates the first investigation of detection of leptin receptor expression in highly purified β-cells. Previous studies have confirmed leptin receptor expression in islets with the less sensitive method of RT-PCR [173-176, 178-180, 182, 185, 190, 209].

We found that levels of leptin receptor mRNA and of leptin signalling are vanishingly low in highly purified mouse islet cells and whole islets, respectively (Figure 3.14). These results corroborate work from Fujikawa, Coppari and colleagues [210] who showed, through the use of a leptin receptor promoter driving *Cre* crossed to Rosa26tdRFP reporter mice, that RFP expression (reflecting the activity of the LepRb promoter) is essentially undetectable in the endocrine pancreas, both during development and in adult mice. In contrast, recombination was detected in a more poorly-defined cell population outside of the islet, possibly corresponding to duct cells or ductal precursors. Importantly, the latter cells are likely to contaminate islets after isolation, and, as such, may reflect the major pool of LepRb-expressing cells in these *ex vivo* preparations. Consequently, the use of *Cre* deleter strains active throughout the pancreas, notably Pdx1*Cre*, is expected to cause recombination and loss of LepRb from these contaminating (non-islet) cells. The loss of LepRb mRNA apparent in islets from the above mice in a previous report [191], therefore, seems likely to reflect deletion from the above population other than from β-cells *per se*. We note that the report by Covey *et al.* [192] using the RIP2*Cre* deleter strain, and therefore expected in the pancreas to delete only in β cells, does not include any data on LepRb mRNA levels in wild type versus null mouse islets. Only measures of DNA recombination, as performed in our own study, were provided (Figure 3.1).

However, for the quantitation of LepRb mRNA expression in isolated islets from Ins1*Cre*LepRb^{KO} or wild type animals, mRNA levels in isolated islets were below reliable quantification levels. Furthermore, the use of RNAseq LepRb mRNA was detected at 0.2 reads per kilobase per million mapped reads (RPMK) in whole islets which is at the lower 39th percentile of all messages [384]. In purified α, β and δ cells the RPMK was 0, 0.23 and 0.036, respectively (n = 4, 3 and 2 animals respectively, prepared separately).

Further supporting our result of low levels of LepRb in β-cells is a research paper from Segerstolpe and colleagues [211] using dispersed FACS purified pancreatic cells from human donors and demonstrating using single cell RNASeq that LepRb is not expressed in α-,β-,γ-cells acinar cells and ductal cells (Appendix

3). However, in contrast to our own results, these authors reported that pancreatic δ-cells have relatively higher expression of LepRb. A similar result was also obtained observed by Baron and colleagues [212].

3.3.2 Effect of β-cell Leptin receptor signalling on glucose homeostasis

Consistent with earlier findings using more "leaky" *Cre*s [191, 192], we show that the levels of leptin receptors on β cells may be sufficient to restrict β cell function *in vivo* under some circumstances. Thus, we report mild glycaemic effects of LepRb deletion which appeared transiently in female mice (Figure 3.6B). Although challenged by the findings of Coppari and colleagues [210] (above) one possibility, not explored here, is that this might reflect a transiently higher level of expression of leptin receptors on β cells at this developmental stage in females. However, the alternative possibility that a very low level of LepRb expression, perhaps on a minority of well-connected ("pacemaker" or "hub") β cells [431], is sufficient to exert islet-wide consequences on insulin secretion in juvenile females, cannot be discounted.

We note that, whilst in one previous report [192] the effects of β cell-targeted LepRb deletion were examined using oral glucose tolerance tests (OGTTs), where peak blood glucose was increased from 12.5 to 27mM (54%) and from 11 to 16mM (31%) in 6 weeks old males and females, respectively. In a second paper [191], equally dramatic effects of deleting these receptors throughout the pancreas were observed using IPGTTs, where peak blood glucose was reduced from ~22 to 16mM (27 %) and from >16 to ~9.5 mM (40%) in 6 month old males and females, respectively.

Our own experiments, using the more selective Ins1*Cre* driver line to delete LepRb, revealed a much more modest reduction in peak glucose during IPGTT, from ~12 to 10 mM (16 %) in 8 week old females, whereas no differences were observed between control and knockout male mice of the same age, or between 4 month old mice of either sex (Figure 3.6). The measurements in the present study are thus best compared to the latter report [191], using IPGTTs, and support our contention of a limited role for LepRb in β-cells beyond juvenile stages. The more marked effects of LepRb deletion described in earlier studies [191, 192] would seem to be attributable, at least in large part, to the loss of leptin receptors at other sites, notably the brain, with consequent effects on food intake (and hence insulin sensitivity) [192] or potentially in other non-endocrine pancreatic cells [191]. Interestingly, Morioka *et al.* [191] did not observe changes in body weight after Pdx1*Cre*-mediated deletion, whilst markedly elevated insulin secretion and β-cell mass were reported.

There is a possibility that the research result from Morioka and colleagues are due to ablation of the LepRb in pancreatic δ-cells with the Pdx1*Cre* being expressed in these endocrine cells. As mentioned above, LepRb has been demonstrated with RPKM to have enriched expression in δ-cells of human islets [211] (Appendix 2). The expression of LepRb in pancreatic δ-cells is further supported, by co-localisation

of δ-cells (SSR staining) to LepRb in human islets (Appendix 3). The δ-cells comprise 3-10% of the adult mouse islet and even though they are a small population of endocrine cells they are scattered evenly through the islet. Consequently, it is possible that leptin can mediate its effect on glucose homeostasis via the LepRb highly expressed on this endocrine cell type. The scattered δ-cells could in turn via paracrine and endocrine effects signal to other endocrine cells to regulate glucose homeostasis. There is a possibility that these cells function as well-connected "hub" δ-cells exerting effects on insulin secretion. However, there is also a possibility that there are differences in LepRb expression in δ-cells between rodent and human, considering that our own studies did not reveal a high expression in purified mouse δ-cells as assessed by RNA-Seq.

A potential concern with the present studies is that deletion may not have occurred throughout the β cell complement, as we have assumed [384]. Whilst LepRb mRNA levels were too low to quantitate accurately either in LepRb^{F7F} or Ins1*Cre*LepRb^{KO} islets, we believe this possibility to be unlikely. Firstly, we observed clear recombination at the genomic level in isolated islets (Figure 3.1). Secondly, Ins1*Cre* leads to highly efficient recombination at *Lox*P sites in other targets including in LKB1/STK11, AMPKα1 and α2 alleles [384], at the Rosa26-eYFP locus [422], and in mice carrying a single halorhodopsin allele downstream of a *Lox*P-STOP-*Lox*P cassette (G. Rutter, D. Hodson, personal communication). In the two latter models, recombination is observed in 97.8% and 94.7 % of β cells, respectively.

Further supporting a limited role of LepRb on β cells are studies by Chua and colleagues [194] demonstrating an absence of metabolic phenotype in mice deleted selectively for LepRb in the periphery. In light of the above observations, our own data in Ins1*Cre*LepRb^{KO} animals reporting only a weak metabolic phenotype are not entirely unexpected.

Interestingly, the inhibitory actions of leptin observed in the present study appear to be independent of glucose-induced increases in intracellular Ca^{2+}, since the latter showed only a non-significant tendency to be impaired in Ins1*Cre*LepRb^{KO} mice (Figure 3.10). Secondly, the tendency for intracellular Ca^{2+} excursion to be impaired is conflicting to the reported by Morioka were they observe an significantly increased Ca^{2+} excursion and with our result of an enhanced insulin secretion in isolated islets in females as shown in figure 3.9.

3.3.3 Other possible routes of leptin to control glucose homeostasis

It is very possible that leptin's effect on glucose homeostasis is acting exclusively via the brain. Several research studies have shown that areas where the RIP2*Cre* and Pdx1*Cre* promoter are expressed are involved in peripheral glucose regulation. Pdx1*Cre* was found to be active in medial preoptic area (MPA), ARC, DMH, LHA and the inferior olivary nucleus (ION), and with several fibres projecting to the PVN [193, 376]. These brain areas have all been demonstrated to be involved in energy balance and glucose

homeostasis [330, 331, 432, 433]. In the lateral hypothalamus area (LHA) Pdx1*Cre* is expressed in a pattern that is partially overlapped with both the orexin-expressing and neuronal populations and neurons where leptin have been demonstrated to activate pSTAT3 signalling [193].

The orexin neurons in the LHA are involved in feeding regulation and energy homeostasis. Orexin expressing neurons in the LH have been demonstrated to be inhibited by leptin [434]. Orexin knockout mice have normal body weight and when aged they gain an impaired glucose tolerance [435]. This suggests that, deletion of the LepRb with the Pdx1*Cre*-promoter might target these neurons or important subpopulations in the area involved in regulation of glucose homeostasis that in turn regulate insulin secretion by β-cells.

Further supporting the effect of leptin on glucose homeostasis independent on LepRb signalling in the periphery are several research studies that have demonstrated that blood glucose levels in rodent diabetes models are normalized after central administration of leptin with doses that have no effect if they are being administrated peripherally. Thus, Hidaka and colleagues observed that after ICV injection of leptin in streptozotocin–induced diabetic (STZ-D) rats animals regained normal blood glucose levels, an effect that was not seen when the same dose was administered peripherally [436] and repeated by [437] and in mice [438]. The central leptin treatment in these studies did not affect plasma insulin levels which the majority of studies with isolated islets have suggested. This suggests leptin's effect to lower blood glucose is mediated via the brain and not via pancreatic insulin secretion.

3.3.4 Summary

With the use of a highly selective *Cre* recombinase we demonstrate that leptin signalling plays a very minor, age and sex-depended role in the control of β-cell function in mice. Furthermore, leptin receptor mRNA levels were below detection in highly purified β-cells and leptin induced Stat3 phosphorylation was undetectable in isolated islets.

The results from this study suggests that the phenotypes observed in the RIP2*Cre* and Pdx1*Cre* mediated ablation of leptin receptor animals is not due to the loss of leptin receptor signalling in pancreatic β-cells but, more probably, due to a loss of LepRb signalling important areas of the brain involved in energy homeostasis.

In the case of the RIP2*Cre*-mediated ablation, the phenotype observed could be in large part be a phenotype due to obesity these animals are suffering from. Although Covey *et al.* isolated islets and demonstrate impaired insulin secretion; the impairment could also be due to the obesity as they as they confirm defects in the β-cell mass as well. It is not possible to determine if it is obesity that damaged the insulin secreting β–cells or a proposed lack of LepRb signalling in β–cells.

Chapter 4 Leptin's role in GLP-1-neurons, pancreatic α-cells and other proglugacon expressing cells

Abstract

Background and aim: Leptin signals to the brain to inform on energy balance and the disruption of leptin signalling results in hyperphagia and obesity. Leptin receptors are distributed in several areas mainly in the hypothalamus. The function of leptin receptors outside hypothalamus is less explored and the function of LepRb in GLP-1-expressing neurons in the NTS has not been determined.

Methods: In this study, we produced mice deleted for LepRb in GLP-1 neurons, α-cells and other proglucagon cells using mice with a *Cre* under the control of a preproglugacon promoter and mice bearing *flox'd* alleles of LepRb exon 17, required for leptin receptor signalling. Genomic DNA from olfactory bulb, islets and NTS was isolated to confirm the excision of exon 17. Intraperitoneal glucose tolerance tests, insulin tolerance tests were carried out in these mice and body weight progression was followed for 30 weeks. Glucagon secretion was measured both *in vivo* using ELISA and *in vitro* using HTRF.

Results: Deletion of LepRb from α-cells, a minority of β-cells, and a subset of proglucagon-expressing cells in the brain, exerted no effects on body weight, glucose or insulin tolerance, or on pancreatic hormone secretion assessed *in vivo* and *in vitro.*

Conclusions/Interpretation: In this study, we found that LepRb signalling in GLP-1 expressing neurons are not responsible for body weight control nor does it affect glucose homeostasis. LepRb signalling in α-cells does not affect glucose homeostasis or glucagon secretion.

4.1 Introduction

4.1.1 Leptin receptor in GLP-1 expressing neurons of the nucleus tractus solitarius (NTS)

Much is known about leptin receptor's effect on satiety acting on the receptors expressed in hypothalamus, however, less is known about leptin receptors expressed in extra-hypothalamic areas. The two distinct neuronal populations in the ARC of hypothalamus POMC-neurons and NPY/AgRP-neurons both express LepRb and are either inhibited or activated by leptin [439]. Leptin acting on these neurons decreases food intake [439]. However, the action of these neuronal circuits in hypothalamus cannot solely account for the effects of leptin. Firstly, deletion of the LepRb in AgRP and POMC or POMC neurons only results animals with a modest obesity and not reflecting the obese phenotype observed in *db/db* mice [164, 165]. Secondly, these animals are not infertile and do not have hypercorticolism as observed in *db/db* mice. Furthermore, even though there is a high density of expression of leptin receptor in the hypothalamic nuclei they only represent a portion of the total LepRb expression in the brain and several extra-hypothalamic areas expressing LepRb exists (section 1.5.5.1).

One of these extra-hypothalamic sites expressing LepRb is the nucleus tractus solitarius (NTS). NTS is an area located in the brainstem that via vagal afferent neurons receives signals from the gastrointestinal origin and communicates with brain areas such as hypothalamus [440, 441]. Injection of leptin in the periphery results in an activation of leptin signalling in the NTS in mice [430] and rats [295, 298, 442]. Central administration of leptin into NTS in mice results in a fast reduction in food intake [298].

Disruption of LepRb signalling in NTS with a Phox2B deleter strain by Scott and colleagues [302], resulted in hyperphagic animals but with no substantial difference in body weight due to an increase in energy expenditure. A few of the targeted neurons were GLP-1-expressing neurons suggesting that the GLP-1 specific neurons could be involved in food intake regulation via leptin signalling. Furthermore, Trapp and colleagues have shown that leptin directly depolarizes GLP-1-expressing neurons [301].

4.1.2 GLP-1 neurons and glucose homeostasis

Leptin signalling in neurons of the arcuate nucleus has been demonstrated to affect peripheral glucose homeostasis [432]. NTS might also play a role in glucose regulation by affecting vagal-dependent hepatic glucose production via hypothalamus [443]. In addition, it has been discovered that GLP1-R in the hypothalamus is involved in glucose regulation [291, 292]. Therefore it is a possibility that leptin signalling in GLP-1 neurons in the NTS is involved in the control of glucose homeostasis.

4.1.3 Leptin receptor in pancreatic α-cells and L-cells

Preproglucagon is expressed in GLP-neurons, pancreatic α-cells and L-cells in the gut (Figure 1.3) [118]. The pancreatic α-cells synthesize and secrete glucagon and have an important role in controlling glucose levels. Earlier animal studies have ablated the LepRb in α-cells with a less efficient *Cre*-driver strain, the PPG.*Cre* [374] and reported that a partial ablation of LepRb in mice exerted no effect on glucose tolerance [100]. However, studies in isolated islets have demonstrated an inhibitory effect on glucagon secretion upon treatment with leptin.

L-cells are enteroendocrine cells that are located in the epithelia cell lining of intestinal wall in the distal gut. These enteroendocrine cells represents less than a 1% of the cell lining but have a very important function in secreting several different gut hormones involved in energy metabolism. The L-cells synthesise and secrete the gut hormones GLP-1, PYY, oxyntomodulin and GLP-2. GLP-1 is an incretin involved in stimulating insulin secretion and gastric emptying. PYY and oxyntomodulin are both involved in inhibition of food intake. These gut hormones are all secreted postprandially and it is possible that signalling from the leptin receptor could be involved in the regulation of secretion of these hormone and disruption of signalling in these cells might affect food intake.

In this chapter, we delete the leptin receptor in the preproglucagon expressing cells (GLP-1 neurons, L-cells and α-cells) and explore LepRb signalling role on body weight progression and pancreatic hormone secretion.

4.2 Results

4.2.1 Creation of GLP-1 neurons, α-cell and proglucagon-expressing cells LepRb^{KO} mice

To investigate LepRb function in α-cells, GLP-1-expressing neurons and other proglucagon-expressing cells, we ablated LepRb signalling selectively the GLP-1 neurons, as well as in intestinal L-cells and pancreatic α-cells, with the use of a proglucagon (*iGlu*) promoter driving *Cre* [404]. Mice harbouring *flox'd* alleles of exon 17 of LepRb [195] were bred to animals containing the iGlu*iCre* driver [404].

To confirm firstly the deletion of LepRb in targeted cells, genomic DNA was harvested from different tissues and brain areas containing the preproglucagon expressing cells such as the olfactory bulb, NTS, distal gut (containing the L-cells), islets and control tissues of liver and hypothalamus. PCR was performed using a two-set of primer pair, a and c, flanking the *LoxP* sites as indicated in (Figure 4.1A).

As expected, all tissues examined in control animals (LepRb$^{F/F}$) and animals deleted for the LepRb (iGlu*iCre*LepRb^{KO}) contained the larger band of 625bp representing the *flox'd* intact allele as demonstrated in Figure 4.1B. The presence of the additional band representing the deletion of exon 17 (the recombined allele) was clearly detected in islets, and to a lesser extent in the olfactory bulb, the nucleus tractus solitarius (NTS) and gut but absent in control animals and control tissues, Figure 4.1B.

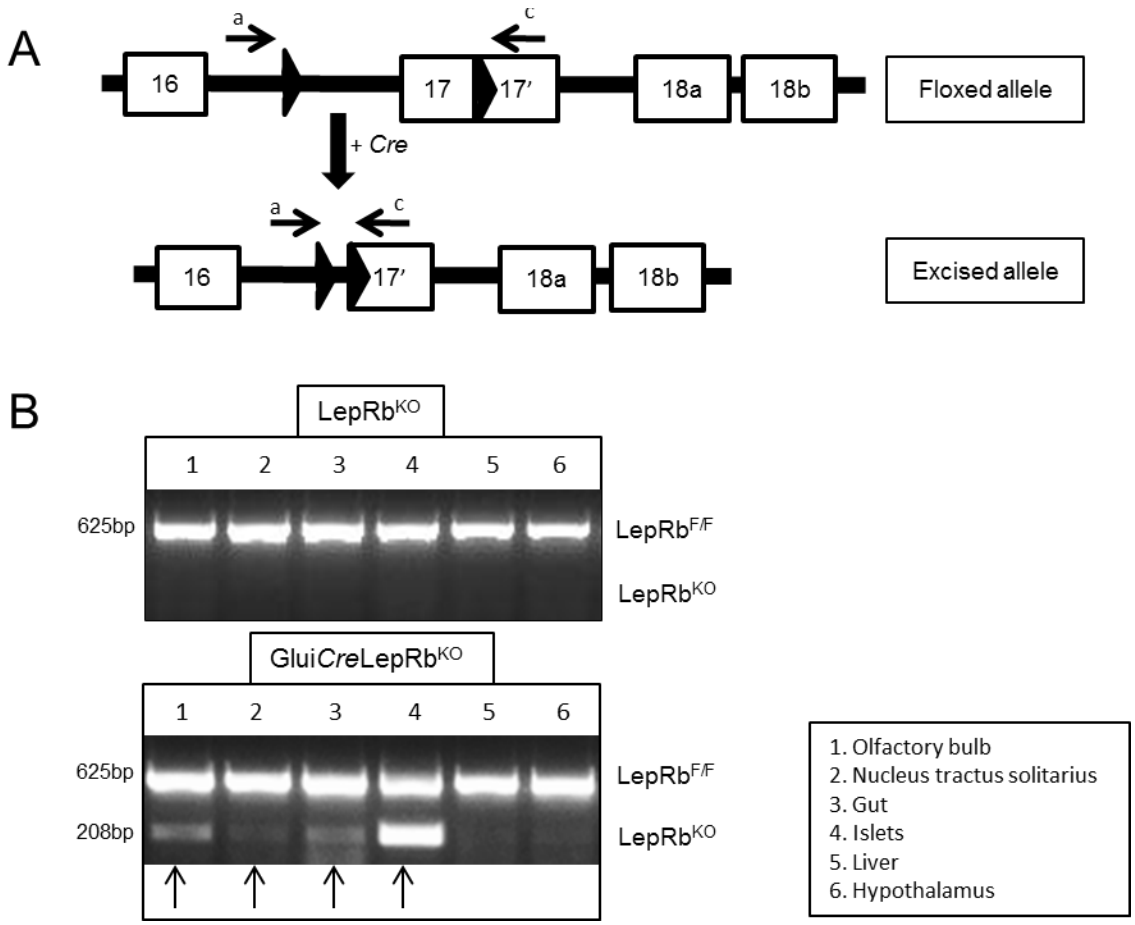
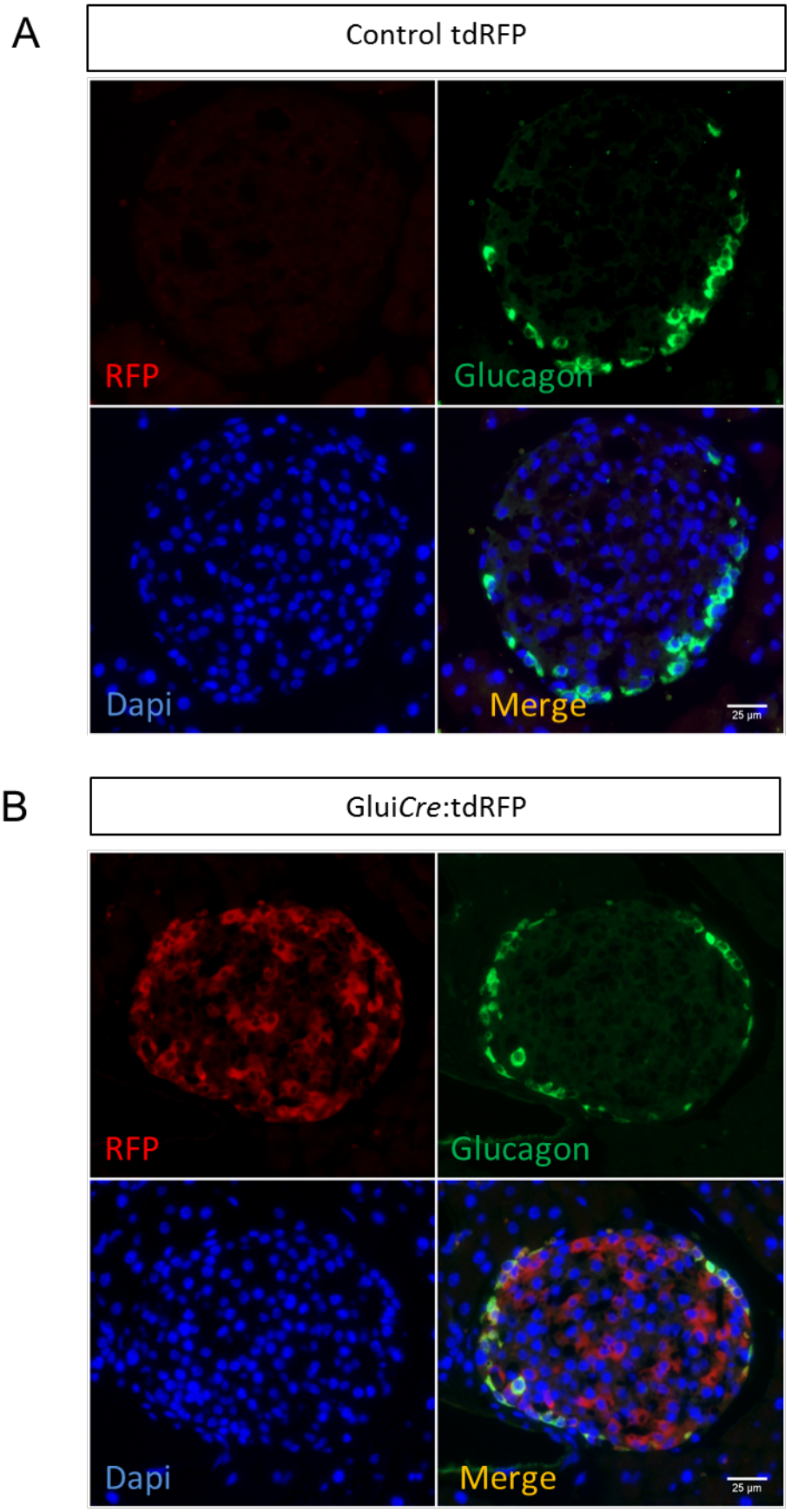


Figure 4.1- iGlui*Cre* expression results in recombination of the *flox*'d region of LepRb alleles selectively in the olfactory bulb, nucleus tractus solitarius, intestine and pancreatic islets. Genomic DNA was harvested from olfactory bulb, nucleus tractus solitaries, gut, islets, liver and hypothalamus and used as a template for PCR. Primer pair a and c are as indicated in (**A)** with forward primer a binding in front of the LoxP site and reversed primer c binding upstreams of the excised allele. Squares denotes exons and black triangle indicates where the loxP sites are located.Predicted product sizes are 625bp for the flox'd allele and 208bp for the excised allele. **(B)** PCR transcript of the excised alleles in and LepRb$^{F/F}$ controls iGlu*iCre*LepRb^{KO}. The arrows are indicating the tissues containing the recombined allele.

To further examine the iGlu*iCre* expression we used the Rosa26.tdRFP reporter mice [394] to investigate the recombination in the pancreatic islets. Efficient *Cre*-mediated recombination leads to expression of the reporter gene tdRFP from the ROSA26 locus (as explained in section 1.8.5). Control animals (Rosa26.tdRFP only) and iGlu*iCre.*tdRFP animals were terminated and the pancreases was taken out,

sectioned and stained for tdRFP, glucagon or insulin. Control animals, that contained no *Cre* but the Rosa26.tdRFP allele, showed as expected no expression of tdRFP (Figure 4.2A), demonstrating that the tdRFP reporter gene is not expressed without *Cre* expression. In mice expressing iGlui*Cre* and carrying the Rosa26.tdRFP reporter, red fluorescence was clearly visible in the majority of glucagon-positive α-cells (green), figure 4.2B. Red fluorescence (tdRFP) was also absent in the majority of insulin-positive β-cells, figure 4.2C.


A
Control tdRFP
RFP
Glucagon
Dapi
Merge
25 µm
B
GluiCre:tdRFP
RFP
Glucagon
Dapi
Merge
25 µm

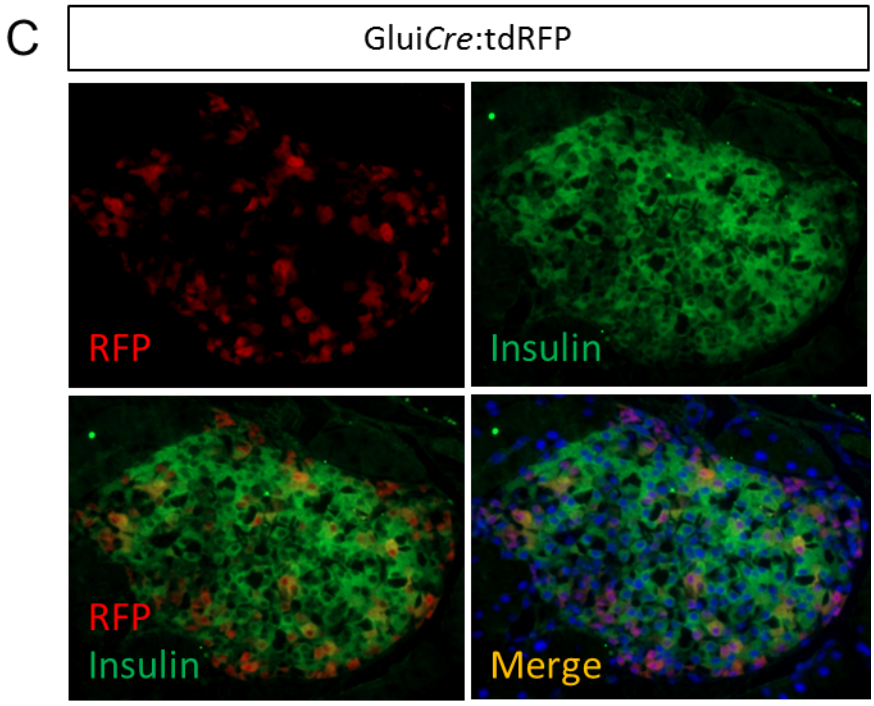


Figure 4.2 - iGlu*iCre* expression results in *Cre* mediated expression of tdRFP. Immunohistochemical analysis of pancreas from control animals without *Cre* and tdRFP^{StopFlox} **(A)** and iGlu*iCre*-tdRFP^{StopFlox}**(B)** mice stained for tdRFP (red), glucagon (green) and DAPI (blue) and (C) mice stained for tdRFP (red), insulin (green) and DAPI (blue)

Considering several tdRFP expressing pancreatic cells did not contain glucagon, cells in islets that co-expressed both glucagon and tdRFP and cells containing tdRFP but not glucagon was counted (islets *n*=39) to investigate the percentage of co-localisation. In the presence of the *Cre* transgene, recombination occurred in the majority (78%) of glucagon expressing islet cells. However, also in a minority (~20%) of non-glucagon positive β-cells expressed tdRFP suggesting that the promoter is somewhat "leaky", figure 4.3.

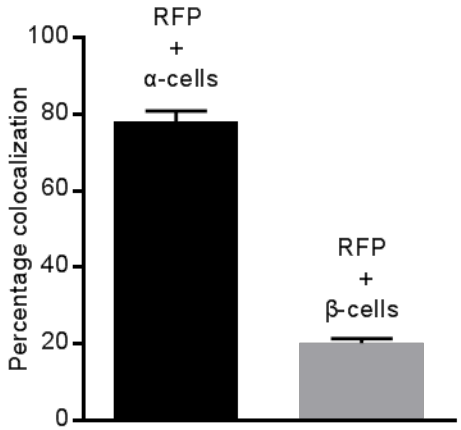


Figure 4.3-The expression of iGlui*Cre* resulted in recombination in the majority of glucagon positive cells. Quantification of the percentage of tdRFP expressing α and β-cells. *n* = 39 islets from three animals. RFP and glucagon (α-cells) Data are expressed as mean ± SEM.

4.2.2 Deletion of LepRb in GLP-1 neurons exerted no effect on body weight progression

The GLP-1 neurons are located in an area involved in energy homeostasis and body weight regulation and several research reports have demonstrated that leptin signalling is involved to mediate these effects [298, 299, 444, 445].

Given that Scott and colleagues observed an effect on body weight in their animals where the leptin receptor is deleted in NTS and are targeting a few GLP-1 expressing neurons have been implicated to have an effect on energy homeostasis [302], we investigated our iGlu*iCre*LepRb^{KO} for body weight progression. Animal's body weight was followed weekly from the age of weaning to 30 weeks of age. We failed to observe differences in weight gain between wild-type and iGlu*iCre*LepRb^{KO} mice (Figure 4.4).

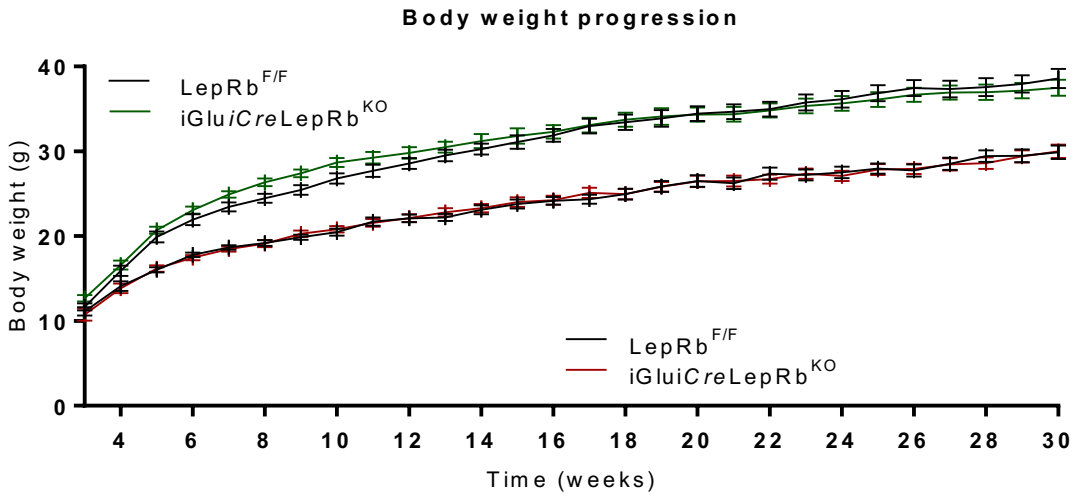


Figure 4.4 - Ablation of LepRb in GLP1-expressing cells does not affect progression in bodyweight. Body weight followed for 30 weeks. Males iGlu*iCre*LepRb^{KO} (green line) and littermate controls LepRb$^{F/F}$ (black line) and below females iGlu*iCre*LepRb^{KO} (red line) and LepRb$^{F/F}$ (black line), (n = 12-19 mice per genotype and gender). Data are expressed as the mean ± SEM and statistical comparison was through two-way ANOVA.

The GLP-1 neurons were examined for their ability to signal via LepRb (pSTAT3). To localize the GLP-1 neurons in NTS iGlu*iCre*:tdRFP animals were stained for tdRFP in addition to pSTAT3. Animals were fasted overnight and injected with recombinant leptin (5mg/kg) to induce pSTAT3 signalling and perfused fixed to preserve the structure of the brain. Brains were sliced and stained for tdRFP representing the GLP-1 neurons and pSTAT3 (LepRb signalling) in the NTS. The majority of GLP-1 neurons (tdRFP) also co-localized with pSTAT3 staining in the NTS (Figure 4.5).

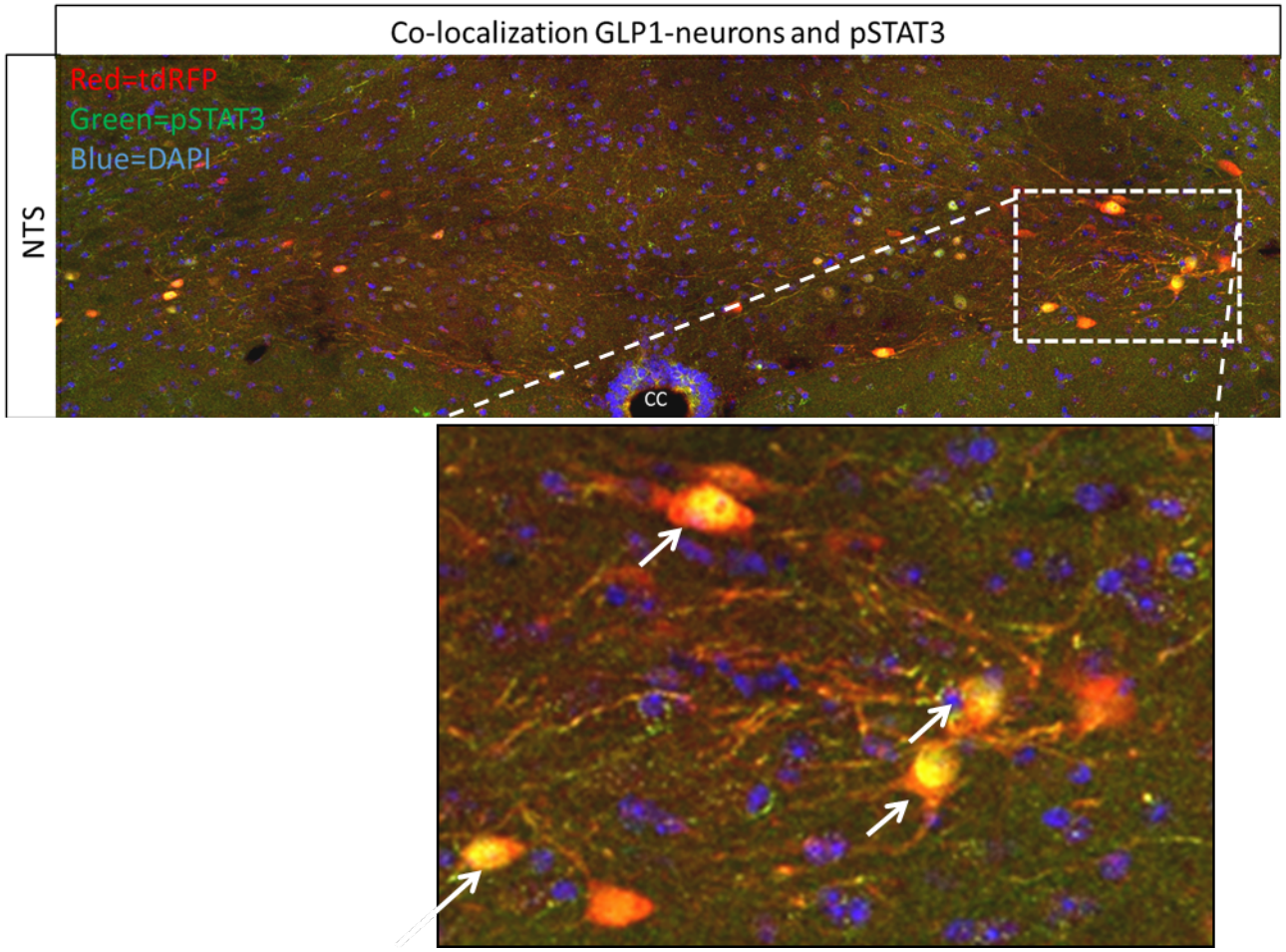


Figure 4.5 - Fasted animals showed STAT3 phosphorylation upon administration of leptin. Immunofluorescence staining of pSTAT3 in the NTS from fasted iGlu*iCre*:tdRFP animals injected with recombinant leptin (5mg/kg). Red=tdRFP, Blue=Dapi, Green=pSTAT3. Arrows are indicating the GLP-1 expressing neurons co-localizing with the induced pSTAT3 signalling. CC=central canal.

4.2.4 LepRb signalling in α- and proglucagon-expressing cells has no effect on glucose homeostasis

Glucagon plays an important role in maintaining the blood glucose levels in the physiological range and leptin has been demonstrated to have an effect on glucose homeostasis. Leptin has also been demonstrated to have an inhibitory effect on α-cells in mice and human isolated islets [99]. Secondly, L-cells in the gut secreting for example GLP-1 are well known for affecting the glucose levels. It is also possible that LepRb signalling in the GLP-1 expressing neurons located in the NTS might have an effect on glucose homeostasis considering GLP-1 expressing neurons are involved in glucose regulation [291].

Therefore we set out to investigate if an ablation of LepRb with iGlu*iCre* transgene would affect the blood glucose levels. Animals iGlu*iCre*LepRb^{KO} and littermate control LepRb$^{F/F}$ at the age of 8 weeks was fastend

overnight and blood glucose levels measured. No difference was observed between the two different genotypes (Figure 4.6 A,B).

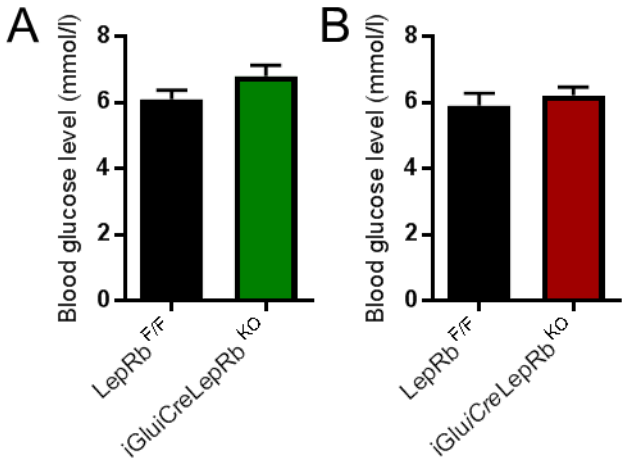


Figure 4.6 - No difference in fasting blood glucose levels was observed in iGluCre mediated deletion of LepRb. Animals at the age of 8 weeks were fasted O/N and blood glucose measured**. (A)** Blood glucose in male control animals LepRb^{KO} black bar, *n*=11 and iGlu*iCre*LepRb^{KO} green bar, *n*=6. **(B)** Blood glucose in female control animals LepRb^{KO} black bar, *n*= 6 and iGlu*iCre*LepRb^{KO} green bar, *n*=8. Data are expressed as the mean ± SEM.

Male and female animals at the age of 8 week deleted for the leptin receptor with iGlu*iCre* transgene were also tested for their ability to clear blood glucose after an IP injection of glucose. The different genotypes revealed no difference in their response to glucose neither in males (Figure 4.7A) nor in females (Figure 4.7B). Animals were also challenged with insulin and their glucose levels measured but no differences were apparent (Figure 4.8A,B).

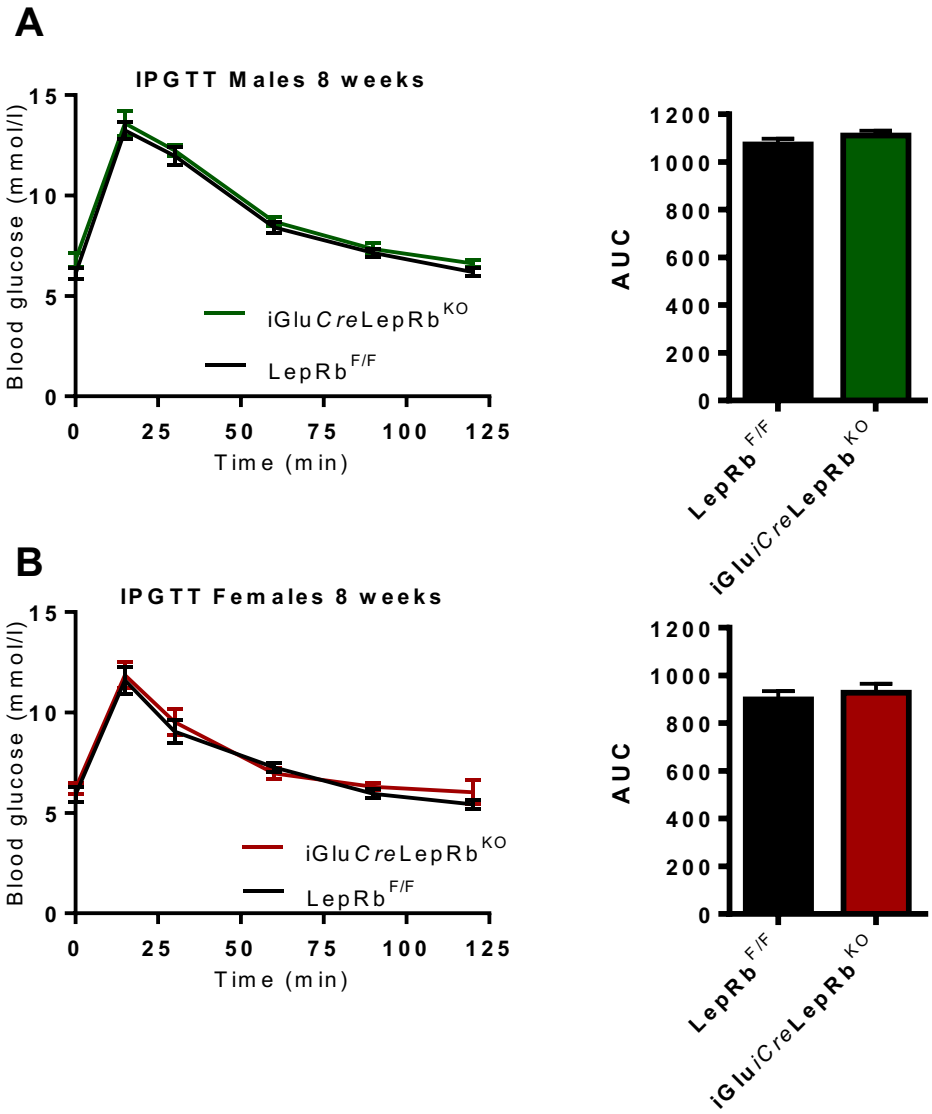


Figure 4.7 - iGlui*Cre*LepRb^{KO} animals display normal glucose tolerance at the age of 8 weeks. (A) Blood glucose concentration following IPGTT in male iGlui*Cre*LepRb^{KO} (green line) and littermate control LepRb$^{F/F}$ (black line) in 8 week old animals: iGlui*Cre*LepRb^{KO}, *n*=6 and LepRb$^{F/F}$ *n*=11. Inset is area under the curve. **(B)** IPGTT in female iGlui*Cre*LepRb^{KO} (red line) and littermate control LepRb$^{F/F}$ (black line) in 8 week old animals: iGlui*Cre*LepRb^{KO}, *n*=8 and LepRb$^{F/F}$ *n*=6. Inset is area under the curve. Data are expressed as the mean ± SEM and statistical comparison was through two-way ANOVA or student t-test.

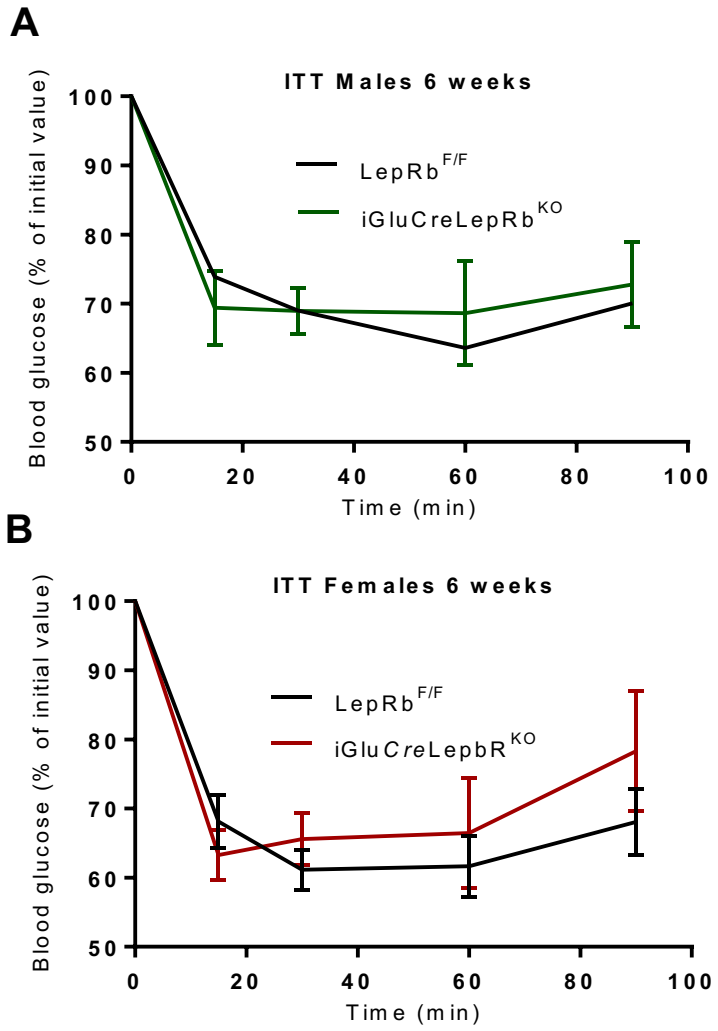


Figure 4.8 - iGlui*Cre*LepRb^{KO} animals display normal insulin tolerance at the age of 6 weeks. (A) Blood glucose concentration after ITT in males: iGlu*iCre*LepRb^{KO}, *n* = 8, LepRb$^{F/F}$, *n* = 7; and females (B): iGlu*iCre*LepRb^{KO}, *n*=8, LepRb$^{F/F}$, *n*=10. Data are expressed as the mean ± SEM.

Considering that leptin affected glucagon secretion by direct inhibition on islets and glucagon-expressing cell lines, animals were challenged with insulin and blood samples taken for glucagon measurements. We failed to see a difference in plasma glucagon levels detected with an ELISA kit during hypoglycemia between animals deleted for the LepRb and their littermate controls, Figure 4.9. These findings are consistent with findings from Tuduri and colleagues [100] who failed to see any effect on a partial ablation of leptin receptor.

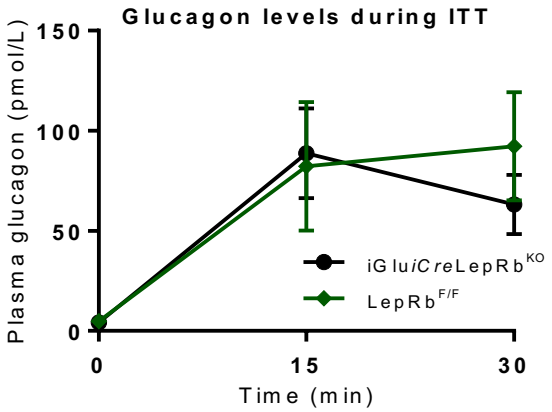


Figure 4.9 - Attenuation of the leptin receptor in proglucagon expressing cells does not affect glucagon secretion *in vivo*.
Plasma glucagon levels during insulin tolerance test iGlu*iCre*LepRb^{KO}, *n*=6, LepRb$^{F/F}$, *n*=4. Data are expressed as mean ± SEM.

Leptin treatment of isolated islets has previously been demonstrated to inhibit glucagon release in response to hypoglycemia an effect that was counteracted by the phosphatidyl inositol 3' kinase inhibitor, wortmannin. Therefore, we isolated islets from animals deleted for the LepRb in preproglucagon expressing cells and their littermate controls and measured glucagon release from isolated islets using a HTRF kit. Glucagon release from wildtype isolated islets is usually maximal at low glucose of 0.5mM and reduced (~55%) at high glucose (10mM) [83]. We found that glucagon secretion from iGlu*iCre*LepRb^{KO} mouse-derived islets was reduced, with an 83.38% reduction in control animals at high glucose levels and with a 74% reduction in iGlu*iCre*LepRb^{KO} animals (Figure 4.10).

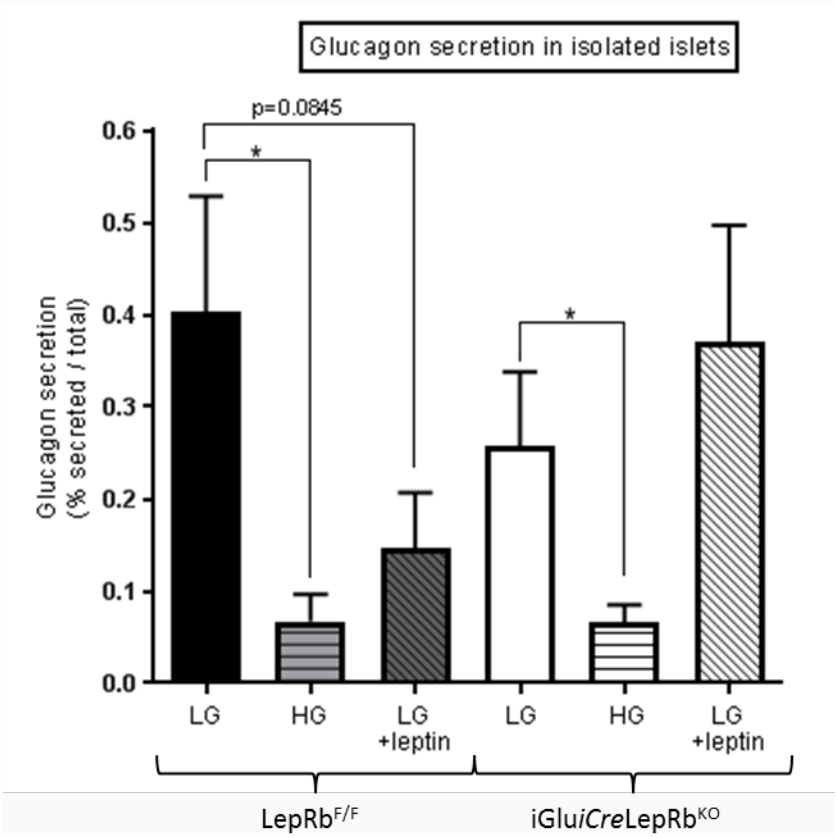


Figure 4.10 - Glucagon secretion in isolated islets after incubation with leptin. Glucagon release from isolated islets in the presence of the indicated glucose concentrations: LG = 0.5 mM, HG = 10 mM glucose; *n* = 6 animals per genotype. When present, leptin was added to 10 nM. Data are expressed as the mean ± SEM and statistical comparison was through 1-way anova.

Given that leptin inhibits glucagon secretion from isolated mouse islets and from a glucagon-expressing cell line [99], we measured glucagon release from isolated islets after 40 min incubation with 10nM recombinant leptin at low glucose levels in iGlu*iCre*LepRb^{KO} and control litter mates LepRb$^{F/F}$ (Figure 4.10). Leptin showed a non-significant (p=0.0845) tendency to impair glucagon secretion in control islets. This tendency to impair glucagon secretion was lost in islets from iGlu*iCre*LepRb^{KO} animals (Figure 4.10).

4.3 Discussion

In this study we sought to unravel the *in vivo* phenotype of disrupting leptin signalling in α-cells, L-cells, GLP-1 neurons to determine if LepRb signalling in these cells are involved in glucose homeostasis or body weight regulation.

4.3.1 Leptin receptor signalling in α-cells

Evidence suggests that leptin inhibits α-cell activity and glucagon secretion [99]. Animals that lack leptin or the leptin receptor have high concentrations of glucagon [446, 447]. Injection of leptin into animals that lack leptin reduces the increased glucagon levels [448]. Using the less efficient PPG.*Cre* [374], earlier studies [100] have reported that elimination of LepRb from a subset of α-cells exerted no effect on glucose tolerance. However, and consistent with findings from Tuduri *et al*. [99], we observed a tendency for leptin to inhibit glucagon secretion *in vitro* (Figure 4.10), an effect which was abolished in iGlu*iCre*LepRb^{KO} mouse islets. Similarly, a tendency was observed towards a more rapid recovery towards normal glycemia after insulin injection in female KO mice (Figure 4.8). Thus, at least under the conditions examined here, leptin receptor signalling in α-cells has no more than a marginal role at most in the control of glucagon secretion during hypoglycemia.

The observation by Tuduri *et al,* where leptin treated islets had a decreased glucagon secretion is at supraphysiological concentration of 10nM could be due to the possibility that leptin at these concentrations are toxic to the islets. Normal plasma levels of leptin in mice are increasing with age and increases with high fat diet in mice. [449]. At aged mice of 2 months leptin levels are around ~0.4nM and exposing isolated islets to high concentration of 10nM could therefore inhibit the α-cells islets by being cytotoxic to the islets.

We and others [175, 211], have failed to detect any evidence of LepRb expression in the α-cell (Figure 3.14B). Our findings are, furthermore, consistent with the use of the LepRb*Cre* transgenic mice by Fujikawa *et al*., demonstrating that the LepRb in α-cells are not expressed at any time in pancreas during development [210]. In human islets, two very recent and independent research studies have demonstrated that LepRb is not expressed on α-cells [211, 212]. Interestingly, both studies revealed that LepRb is expressed on the somatostatin producing δ-cells and there is a possibility that the effect on glucagon secretion observed by Tuduri and colleagues is mediated via these cells. However, very low expression levels have been found in purified mouse islets We notice that in human islets the authors used a lower concentration of leptin (0.625nM) whereas in mice islets a ten times higher concentration was used, demonstrating that human islets are more sensitive to leptin. If leptin inhibits both insulin secretion and glucagon it would mimic the effect of somatostatin that has been demonstrated to have an inhibitory effect on both insulin and glucagon secretion providing a negative feedback [450-452].

Our results suggest that the suppressive effect of leptin administration on glucagon secretion observed by others could not be mediated through direct interaction with the α-cells but possibly by indirect action via the brain or via the pancreatic δ-cells.

4.3.2 Leptin receptor signalling in GLP-1 neurons

Leptin receptors in the hindbrain have been demonstrated to have an effect on food intake and body weight in rats [300]. In addition, Scott and colleagues with Phox2B promoter deleted the LepRb in neurons of the NTS hindbrain and observed an increase in body weight.

In the present study, the iGlu*iCre* promoter allowed us to generate mice deleted for LepRb signalling selectively targeting the GLP-1 neurons. We failed to observe differences in weight gain between wild-type and iGlu*iCre*LepRb^{KO} mice. Whilst the simplest interpretation of these data would be that LepRb in GLP-1-expressing neurons in the NTS are not involved in the control of food intake, we note that Scott and colleagues [302] observed that disruption of LepRb signalling in this brain region with the less selective Phox2B deleter strain led to hyperphagia, but a modest weight gain. This was attributed to an increase in metabolic expenditure in the absent of any change in the locomotor activity in null mice. Whether deletion of LepRb using the iGlu*iCre* transgene similarly affected food intake and/or energy expenditure is a possibility which will require testing with individually-housed mice in metabolic cages in the future.

Leptin acting on the arcuate nucleus have been demonstrated to have an effect on glucose homeostasis [432] and GLP1-R have been shown to play a role in regulation of glucose. In this study, we failed to detect a difference in glucose homeostasis consistent with the result from Scott and colleagues [302].

4.3.3 Leptin receptors in other proglucagon-expressing cells

Intestinal L-cells, which synthesize and secrete GLP-1, are also an important site of proglucagon expression, and consequently recombination in iGlu*iCre* mice [404, 406]. In addition, several other gut hormones involved in energy balance such as oxyntomodulin are expressed and secreted from the intestinal L-cells. Leptin and these gut hormones levels are all increased in response to feeding acting on the same pathways of energy regulation. Although not measured directly in the present studies, changes in GLP-1 or oxyntomodulin output from these cells would seem to be unlikely in iGlu*iCre*LepRb^{KO} mice given their unaltered body weight gain (Figure 4.4) and glucose tolerance (Figure 4.7), as well as the low expression of leptin receptor mRNA in FACS-purified mouse L-cells (Frank Reimann and Fiona Gribble, University of Cambridge, U.K., data not shown) [384].

Chapter 5 LKB1 function in RIP2*Cre*-expressing neurons of the ARC

Abstract

Background and aim: Hypothalamus is a brain area that is involved in the control of feeding and glucose regulation. RIP2*Cre* neurons are located in this area and deletion of LKB1 in these neurons and β-cells together results in animals that are lean, suggesting a role for LKB1 in the brain regulating of feeding. In this study we investigate the role of LKB1 in neurons of the arcuate nucleus (ARC) in hypothalamus.

Methods: In this study, we utilize an AAV2–virus to transfer the gene of *Cre* to ablate LKB1 in RIP2*Cre* neurons of the ARC of hypothalamus. The AAV2 virus contains the mouse insulin 2 promoter (mIP2) driving *Cre* was by stereotactic surgery delivered into the ARC of hypothalamus in LKB1 *flox'd* mice. The mIP2 promoter allows for specific targeting of the RIP2*Cre* neurons. A control virus lacking *Cre* was injected into littermate controls of the LKB1 *flox'd* mice. Animals injected with virus were investigated for changes in body weight and glucose tolerance.

Results: No effect upon deletion of LKB1in RIP2*Cre* neurons was observed in body weight progression followed for 4 weeks post virus mediated deletion. We also failed to observe a difference in glucose tolerance.

Conclusions/Interpretation: Our findings suggest that LKB1 does not play a role in the RIP2*Cre* neurons of the arcuate upon body weight or glucose homeostasis in adult mouse brain.

5.1 Introduction

The hypothalamus contains several regions involved in the regulation of appetite. Unbalanced satiety regulation can lead to obesity, one of the most common medical problems in the developed world, affecting men and women at all ages [453]. The hypothalamus is subdivided into several nuclei and the main one in the regulation of appetite is the arcuate nucleus (ARC). This nucleus contains NPY/AgRP, POMC-neurons and a less defined subpopulation of neurons named RIP2*Cre* neurons. RIP2*Cre* neurons are neurons containing expression of the rat insulin II gene and are proposed to be involved in energy homeostasis.

5.1.1 RIP2*Cre* neurons

As discussed in Chapter 1, rodents have two non-allelic insulin genes, insulin I and insulin II, where insulin II is the human homologue [21, 23, 24]. Insulin II in contrast to insulin I is expressed in the brain [25, 26]. Insulin I expression is confined to β-cells and is expressed at a less degree with 1:2 ratios to insulin II expression [25]. Peripheral synthesised insulin crosses the BBB binding to its receptors expressed in hypothalamus and thereby reducing food intake. The insulin II promoter from rat (RIP2) has been utilized in transgenic mice to drive *Cre* expression in β-cells and is by the additional expression in the brain the RIP2*Cre* neurons was discovered [229]. The neurons are intermingled with NPY/AgRP and POMC-neurons but are distinct from them considering they do not express these peptides [229].

RIP2*Cre*-expressing neurons are a population present in several regions of the brain, but concentrated in the arcuate nucleus of hypothalamus, and to a lesser degree in other hypothalamic regions [376]. However, different promoters gave slightly different expression pattern in the brain [193].

5.1.2 RIP2*Cre* neurons and energy homeostasis

Several research studies have found RIP2*Cre* neurons to have an effect on energy homeostasis [229]. The studies have used the RIP2*Cre* transgene with the initial purpose to investigate the effect of the gene deletion in β-cells. Several deletions were found to have a change in energy metabolism that cannot be attributed solely to the deletion in β-cells [192, 229, 377, 378, 380, 381].

Two research studies have investigated the RIP2*Cre* neurons selectively in the brain. Rother and colleagues deleted all RIP2*Cre* neurons selectively in the brain and acutely in adult mice and found animals to be lean [385]. On the other hand, Kong and colleague deleted the synaptic transmission of GABA and glutamate in RIP2*Cre* neurons and found that deletion of GABA led to animals with an increased body weight that was not attributed to an increase food intake instead a decrease in energy expenditure. The RIP2*Cre* neurons are

located in several areas and the group confirm what specific subgroup that are responsible for the observed phenotype being the GABAergic RIP2*Cre* neurons in the ARC [252].

5.1.3 LKB1 function in RIP2 neurons of the arcuate nucleus of hypothalamus

Previous results from Rutter laboratory have shown that deletion of Liver kinase B1 (LKB1) with the RIP2*Cre* deleter strain, deleting in the brain as well as in β-cells leads to dramatic effect on glucose homeostasis but also a decrease in body weight [366]. In contrast, deleting LKB1 with Ins1*Cre* driver strain selective for β-cells showed no effect on body weight [384], suggesting that the difference in weight is due to deletion of LKB1 in the RIP2*Cre* neurons. LKB1 has been demonstrated to be expressed in the arcuate nucleus [370]. Deletion of LKB1 in the POMC neurons in the ARC results in animals with glucose intolerance and insulin resistance observed in female mice [370].

We therefore decided to investigate the role of LKB1 in the RIP2*Cre* neurons in the arcuate nucleus and investigate its involvement in regulation of body weight and glucose homeostasis. Our aim was to selectively delete LKB1 in the RIP2*Cre* neurons in the arcuate with stereotactic injection of *Cre*-recombinase virus, to be able to determine the β-cell part versus the brain to investigate LKB1's role in feeding behaviour and glucose homeostasis.

5.2 Results

5.2.1 Acute ablation of LKB1 in RIP2 neurons of the arcuate nucleus

To determine the function of LKB1 in RIP2*Cre* neurons, an adeno-associated virus (AAV), harbouring the mouse-insulin2 (mIP2) promoter driving *Cre* was stereotactically injected into the arcuate nucleus in LKB1 *flox'd* animals to ablate LKB1 specifically in the RIP2*Cre* neurons.

To be able to target the arcuate nucleus successfully, stereotactic coordinates were optimized by analysing ink injections into the brain of freshly sacrificed mice carcasses. The stereotactic surgery was performed as described in methods section 2.5.3. Briefly, to target the ARC, mice were placed in a stereotactic frame and AAV2-virus was injected bilaterally into the optimized stereotactic co-ordinates in anesthetized adult male mice.

Given that a decrease in body weight was observed with the RIP2*Cre*-driver ablating LKB1 in β-cells and hypothalamus in mice [366, 382] which was not observed in animals ablated for LKB1 with the Ins1*Cre* driver [384], we followed animal's body weight post-surgery to investigate whether an acute deletion of LKB1 in RIP2*Cre* neurons in the arcuate nucleus with AAV2:mIP2-eGFP-*iCre* would affect this

parameter. In animals injected with either AAV2:mIP2-eGFP-*iCre* or control virus AAV2:mIP2-eGFP body weight was followed for 4 weeks post-surgery (Figure 5.1A-B). The AAV2-virus takes ~5 days until expression is initiated (of *Cre* in our case) and a subsequent deletion of LKB1. In addition, animals need to recover from weight loss during surgery, therefore, body weight was followed from day 1 (day of surgery=0) and percentage of body weight increase was calculated from day 4 post surgery. LKB1$^{F/F}$ mice injected with AAV2:mIP2-eGFP-*iCre* had a normal body weight progression in comparison to animals injected with the control virus AAV2:mIP2-eGFP (Figure 5.1A-B).

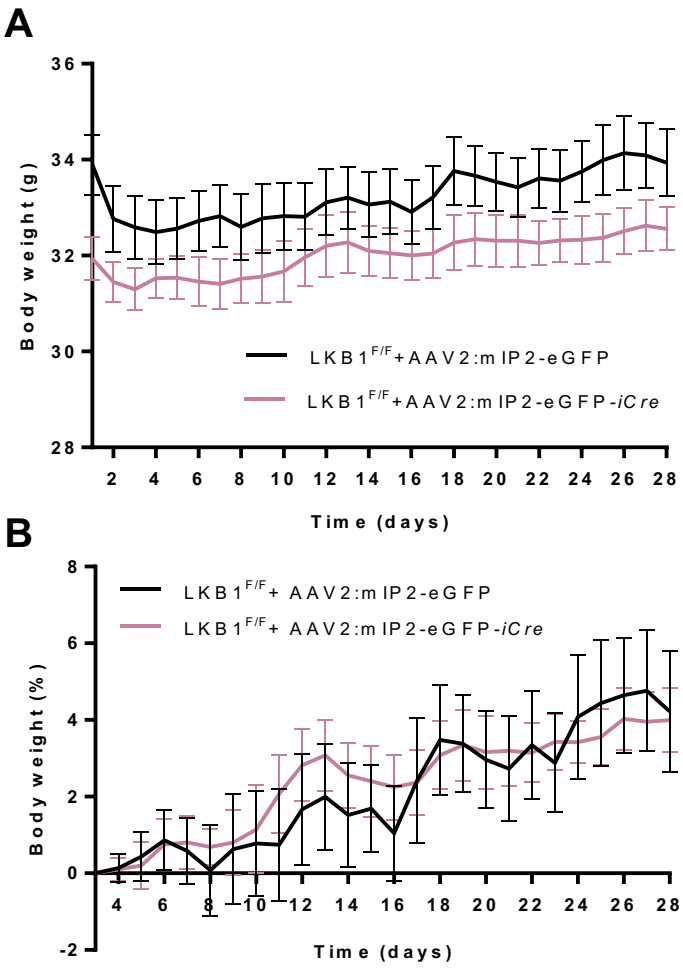


Figure 5.1 - AAV2-*Cre*-mediated deletion of LKB1 in the arcuate nucleus. LKB1$^{F/F}$ mice injected with AAV2:mIP2-eGFP-*iCre* or control virus AAV2:mIP2-eGFP. **(A)** Body weight followed from day 1 (day of surgery=0) to day 28, animals injected with control virus AAV2: mIP2-eGFP, *n*=8 (black line) and animals injected with AAV2:mIP2-eGFP-*iCre*, *n*=10 (pink line). **(B)** Percentage change in body weight calculated from day 4 after injection. Data are expressed as the average ± SEM.

5.2.2 LKB1$^{F/F}$ animals injected with AAV2:mIP2-eGFP-*iCre* displayed reduced fasting blood glucose levels

The ARC of hypothalamus is a brain area not only involved in body weight regulation but contains several neuronal circuits that are part of the control of glucose homeostasis [432] and given that RIP2*Cre* mediated

deletion of LKB1 resulted in animals with an impaired glucose tolerance and reduced fasting blood glucose levels [366], we investigated our animals for fasted blood glucose levels. Mice were fasted overnight and blood glucose measured in the morning 30 days post-surgery. LKB1$^{F/F}$ + AAV2:mIP2-eGFP-*iCre* animals showed a decrease in blood glucose levels in comparison to control animals LKB1$^{F/F}$ +AAV2:mIP2-eGFP (Figure 5.2A). A slight difference in the corresponding fasting body weight was also observed but not significant.

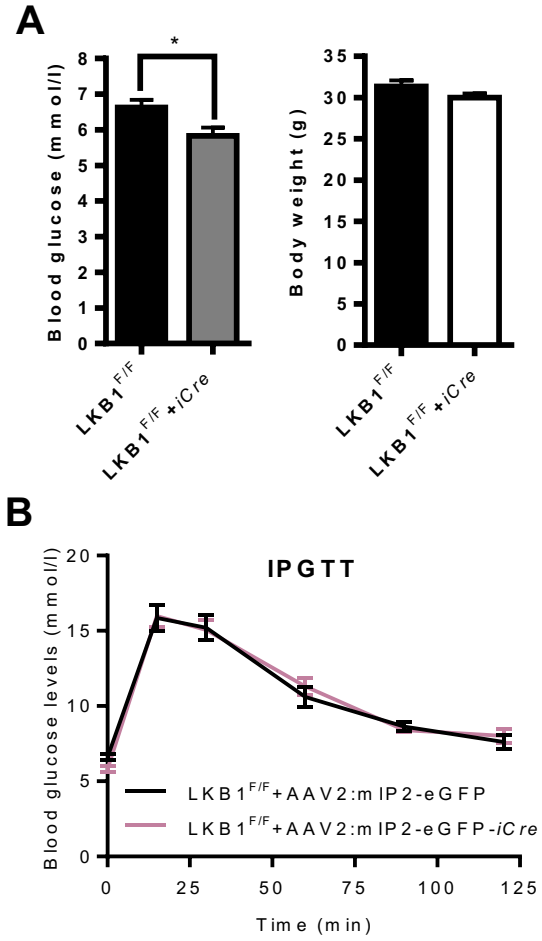


Figure 5.2 - LKB1$^{F/F}$ animals injected with AAV2:mIP2-eGFP-*iCre* displayed decreased fasting blood glucose levels. (A) Blood glucose concentration following overnight fasting in animals 30 days after stereotactic surgery and the corresponding body weight at the time in control animals AAV2: mIP2-eGFP injected, *n*=8 and AAV2:mIP2-eGFP-*iCre* injected , *n*=10. **(B)**. Blood glucose levels after a challenge with 1g per kg mouse glucose. Control animals injected with AAV2: mIP2-eGFP, *n*=8 (black line) and animals injected with AAV2:mIP2-eGFP-*iCre*, *n*=10 (pink line). Data are expressed as the average ± SEM. Statistical analysis was performed using unpaired t-test or two-way ANOVA *P<0.05.

To evaluate if LKB1 ablation in the arcuate nucleus would affect the animals' ability to handle a glucose challenge, animals were subjected to a glucose tolerance test 30-days post-surgery by IP injection of 1g glucose per kg mouse. No difference was observed between control animals and animals deleted for LKB1 in RIP2*Cre* neurons (Figure 5.2B).

5.3 Discussion

In this study, we failed to see an effect on body weight in animals with AAV2-mediated ablation of LKB1 in the RIP2*Cre*-neurons of the ARC in hypothalamus. This is in contrast to the lean animals observed by Sun and colleagues after deletion of LKB1 with the RIP2*Cre* transgene [366] and the total ablation of all RIP2*Cre*-neurons that similarly resulted in lean animals [385]. We also failed to observe an effect on glucose tolerance between animals deleted for LKB1 in RIP2*Cre* neurons and control animals. We noticed a small difference in fasting blood glucose levels with a decrease in animals deleted for LKB1 (figure 5.2A), however this is probably attributed to a slight difference in the body weight, figure 5.2B.

A reason we failed to see a phenotype could be due to that animals stereotactically injected with the AAV2-mIP2-*Cre* virus and control viruses were aged animals and it is possible that LKB1 in RIP2*Cre* neurons of the ARC are only important in this brain area in the developing animal. The RIP2*Cre* promoter is expressed during embryonal stage from day 11.5 (E11.5), [375], and the effects seen by Sun *et al*., in the RIP2*Cre*LKB1^{KO} animals could be due to LKB1 deletion at an earlier age.

The animals deleted for LKB1 with the RIP2*Cre* promoter by Sun and colleagues, also suffer from axon degeneration in the spinal cord and hind-limb dysfunction with an onset of 7 and 8 weeks for females and males, respectively and dies within 1-2 weeks after onset [382]. These other defects the animals are suffering from could be responsible to the lean phenotype observed in these animals and not LKB1 function in the RIP2*Cre* neurons.

Correct targeting of the arcuate nucleus in mice is technically challenging due to its location at the very bottom of the brain and being adjacent to the 3rd ventricle and there is a possibility that the stereotactic virus delivery could have been outside of the arcuate nucleus. Further experiments to confirm correct targeting of the arcuate nucleus with detection of the expression of the marker eGFP expressed from the virus, in addition to experiments to confirm efficient *Cre*-mediated knock-down of LKB1 expression in the RIP2*Cre* neurons would have to be performed.

It is also possible that the fact that we used a fragment of the mouse insulin II promoters instead of fragment of the rat insulin II promoter that has been utilized in the transgenic animal models, the same expression might not be identical in the brain and different neuronal populations could be targeted. However, the sequences between mouse and rat insulin II are almost identical but considering they are both endogenous promoters' regulatory sequences elsewhere in the genome could be different and thereby giving a different expression pattern or even a lack of expression.

In addition, there is a possibility that insulin II is only expressed during the embryogenesis and though even

if LKB1 has a function in the adult brain in feeding regulation if mIP2*Cre* promoter is not expressed at the age of AAV injection our approach will not delete LKB1. Considering peripheral insulin can cross the BBB it is difficult to distinguish centrally produced and insulin with peripheral origin. However, a small study by Devaskar and colleagues [26] did confirm small amounts insulin II expression in CNS with RT-PCR during all stages of the development. In another research study they do see insulin II expression in the brain measured with qRT-PCR, however they did not see expression in hypothalamus [454]. On the other hand, very convincingly Kong and colleague inject *Cre*-dependent viruses in to the ARC of RIP2*Cre* animals at the age of 3-6 weeks demonstrating that at this age RIP2*Cre* is expressed.

Lastly, we could have targeted the wrong specific subgroup of RIP2*Cre* neurons by targeting the ARC RIP2*Cre* neurons. For instance, the destruction of LHA of hypothalamus is an area were a subpopulation of RIP2Cre neurons are located leads to lean animals. It is possible that LKB1 effect on bodyweight is mediated via these neurons. In any case, further studies are needed to more accurately map the sites and efficiency of LKB1 ablation achieved using the approach adopted here

Final Summary and Perspectives

This thesis describes the impact of the disruption of leptin signalling selectively in the pancreatic β-cell and demonstrates a minor change in glucose tolerance in young female mice that were reversed in aged animals. We found that male mice inactivated for leptin receptors in these cells displayed no abnormalities in glucose tolerance.

In addition, we found that blockade of leptin signalling in α-cells, L-cells and GLP-1 neurons had no effect on glucose homeostasis or body weight. Furthermore, and unexpectedly, we found vanishingly low expression of LepRb in whole islets as well as in purified pancreatic cells. This is consistent with several recent findings, published after the work in this thesis was completed and published in part [455], that show no LepRb expression in mouse islets [438] or in highly purified individual human α or β-cells [211, 212]. Our results thus demonstrate that the effect of leptin on glucose homeostasis is not mediated via leptin signalling in the pancreatic α-cells or β-cells. Whether the subtle, gender and age-dependent effects of leptin on insulin secretion and on glucose homeostasis may reflect the existence of leptin receptors on δ-cells – at least in human islets –and subsequently of a cross talk between these and β-cells involving leptin regulation of somatostatin release, is worthy of careful future investigation.

The major findings of this thesis are therefore that:

- β-cell leptin receptors play no or only a minor role in glucose homeostasis.
- α-cell leptin receptors are not involved in responses to hypoglycaemia.
- Body weight maintenance does not require leptin action on GLP-1-secreting cells in the NTS or elsewhere.

We also show that the effects of LKB1 deletion achieved in earlier studies using the promiscuous RIP2*Cre* deleter strain may not be the result of ablation in arcuate nucleus of the hypothalamus, as previously postulated. Thus, RIP2 neurons located in other areas of the brain seem more likely to be involved. However, this tentative conclusion needs to be substantiated with more detailed analysis of the efficiency and sites of recombination affected by the stereotactic injection approach used here to introduce *Cre*-expressing viruses.

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Appendix 1 - Isolation of islets and FACS sorting

Isolation of islets and FACS sorting

Male and female mice above the age of 16 weeks expressing a fluorescent protein (Venus) under the proglucagon promoter [286], or EYFP under the control of the prosomatostatin [427] promoter from mice at the age 8 weeks old mice. Animals were sacrificed by cervical dislocation and the pancreas injected immediately with 5 ml of collagenase V (0.5 mg/ml) in Ca2+ and Mg2+-free HBSS. Pancreata were digested and relaseing the islets that were hand-picked into HBSS complemented with 0.1% fatty acid-free bovine serum albumin (BSA) and 11 mM glucose. Islets were disrupted into single cells by trituration following incubation for 1 min in Ca2+-free HBSS containing 0.1x trypsin/EDTA and 0.1% BSA. Cells were immediately sorted by flow cytometry using a BD Influx cell sorter (BD Biosciences, San Jose, CA, USA) equipped with a 488 laser for excitation of Venus or EYFP. To collect alpha and beta cells, Venus-positive and negative cells were collected, with the latter further subdivided into a population that was large (according to side and forward scatter) and with high background autofluorescence at 530 and 580nm. To collect delta cells, only EYFP positive cells were collected. Cells were collected into RLT lysis buffer (QIAGEN) and frozen on dry ice.The islets purification was performed by Dr Alice Adrianssens.

Appendix 2 - Massive parallel sequencing (RNASeq)

Massive parallel sequencing (RNASeq) whole islets and FACS purified

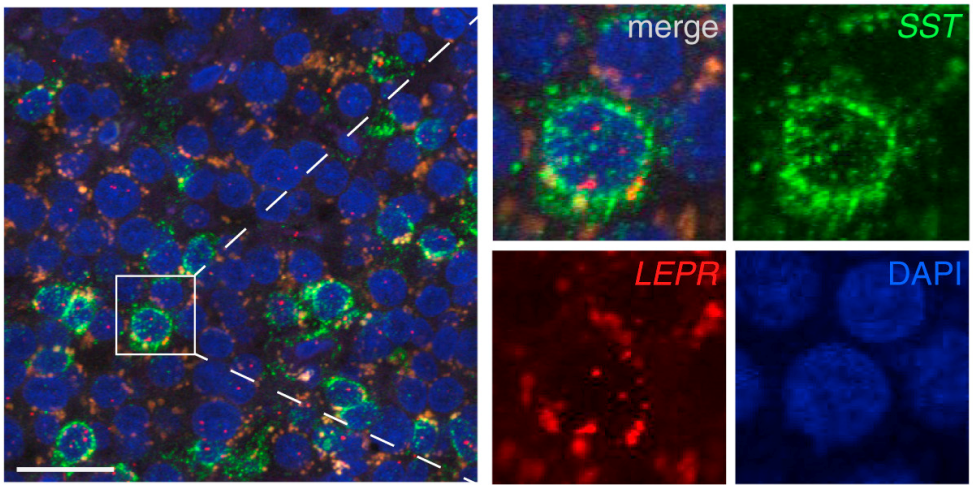
For studies on individual islet cell types, total RNA was extracted from 50–100 wild-type (WT), age 12–14 week. All libraries were prepared using the TruSeq RNA Sample Preparation Kit v2 (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. In brief, magnetic beads containing polydT molecules were first used to purify mRNA from 250 ng of total RNA. Second, samples were chemically fragmented and reverse transcribed into cDNA. Finally, end repair and A-base tailing was performed before Illumina adapters were ligated to the cDNA fragments. Purified samples were amplified by 15-cycle PCR. Amplified material was validated and quantified using an Agilent 2100 bioanalyzer and the DNA 1000 Nano Chip Kit (Agilent, Technologies, Santa Clara, CA, USA). RNASeq for whole islets, and subsequent data analysis, are provided in [384]. LepRb mRNA was detected at 0.2 RPKM in whole islets

For studies on individual islet cell types, total RNA was extracted using an RNeasy Micro Plus kit (Qiagen, Manchester, UK) according to the manufacturer's instructions. RNA was amplified using Ovation RNA-seq System V2 (PN 7102, NuGEN) whereby 2.5ng of RNA for each sample was used (3, 2, and 4 replicates were used each for α, β, and δ cells, respectively, totalling 9 samples). To prepare the RNA-seq library, the amplified cDNA (3µg per sample) was fragmented to 200bp using the Bioruptor Sonicator (Diagenode) and barcode ligation and end repair were achieved using the Ovation Rapid DR Library System (PN 0319,0320, NuGEN). The barcoded libraries were combined and sent for SE50 sequencing using an Illumina HiSeq 2500 system at the Genomics Core Facility, Cambridge Institute, CRUK. 0.23, 0.036 and 0 RPKM in purified β, δ and α and cells, respectively (n=3, 2 and 4 separate preparations, respectively)[455].

Appendix 3 - Single cell transcriptome analysis from human pancreas

Appendix 3 – Single cell transcriptome analysis from human pancreas. Arrow is indicating the RPKM value from LepR. LepRb expression levels are at the 25th lower percentile in α-,β-,γ-cells acinar cells and ductal cells. RPKM values for LepR in δ-cells are showing that the receptor expression is enriched in these cells. Graph is modified from [211]

Appendix 4 - LepRb expression in human δ-cells



Appendix 3 – **LepRb is expressed in human δ-cells**. Human islets stained for LepR (red) and SST (green) and DAPI (blue) demonstrating LepRb expression in δ-cells. [211]

Publications

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1. **Soedling, H**., Hodson, D.J., Gribble, F.M., Reimann, F., Trapp, S. and Rutter, G.A. Limited impact on glucose homeostasis of leptin receptor deletion from insulin or proglucagon-expressing cells. Mol Metab. **4**, 619-630 (2015)

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Abstracts

1. **Soedling, H.,** Trapp, S., Chua, S and Rutter, G.A. Limited impact on glucose homeostasis of highly beta cell selective disruption of leptin signalling. Diab. Med **32** (Suppl 1), 30-206, p97, Abstract P219 (poster, Diabetes UK APC, 2015)

2. **Soedling, H.,** Adriaenssens, A.E., Reimann, F., Gribble, F.M., Hodson, D.J., Trapp, S and Rutter G.A. Limited impact on glucose homeostasis of highly beta or alpha cell-selective disruption of leptin signalling. Diabetologia 58(S1) S278. Poster Presentation 51st meeting of the EASD, Stockholm, 2015