Investigating a role for the corticotropin releasing hormone (CRH) system in the pancreatic islet adaptations to pregnancy

Simpson, Sian

Awarding institution:
King's College London

Investigating a role for the corticotropin releasing hormone (CRH) system in the pancreatic islet adaptations to pregnancy

A thesis submitted by

SIAN J. S. SIMPSON

For the degree of Doctor of Philosophy from
King's College London

Diabetes Research Group
Faculty of Life Sciences and Medicine
School of Life Course Sciences
King's College London

Acknowledgements

I would like to express my gratitude towards a number of people who have had such a positive impact throughout my doctoral training. Dr James Bowe and Professor Peter Jones, I couldn't have asked for a better marriage of supervisors. I am deeply indebted to you James - your door (physically and virtually!) was always open for the many questions I had, and I cannot thank you enough for the countless times you offered an extra pair of hands throughout the *in vivo* studies. Peter, you have given me constant support and guidance, especially when the road got rough and for this, I am extremely grateful. Thank you both for your patience and nurturing. It has been an absolute pleasure to have worked with you.

I also wish to thank Professor Shanta Persaud and Dr Aileen King for their constant encouragement and invaluable advice throughout my studies. I would also like to show my appreciation to Dr Lorna Smith for her mentorship throughout my PhD and all the time she has offered technical and personal support.

My sincere thanks to all of my colleagues within the Diabetes Research Group, both past and present who have played a monumental role in creating and sharing memories with me over the past 4 years. A special thanks to Patricia Fonseca Pedro who has been on this rollercoaster with me from the very beginning. Thank you for all of your support, gin, squashies and most importantly friendship throughout this process. I'm extremely grateful to Dr Elizabeth Evans, Dr Toyosi Bello, Lydia Daniels Gatward, and Dr Olaniru Oladapo for their continuous support both inside and outside of the lab. You have all lent an ear (especially when I have been rambling about my project) and inspired me in many ways to keep going! Of course, I cannot go without acknowledging Dr Inmaculada Ruz Maldonado and Dr Ahmed Arzouni who selflessly offered their time, knowledge and advice to help me navigate through a difficult and scary first year of PhD life. Jai David, you deserve a huge thanks for just putting up with me- despite my cheekiness. Thank you for always being there to help out when I needed anything in the lab.

I am eternally grateful to my friends and loved ones who have always been on stand-by to raise my spirits, cheer me on and provide the occasional/frequent distractions! To the most important people in my life - My Family. Thank you for being my absolute rocks throughout this experience. Mum, you have showered me with unconditional love and support and occasionally provided an Uber service for the early starts and more frequently, late nights. You have held me through my tears (many times) and celebrated with me the small victories over the years. Thank you for all the sacrifices you have made and for teaching me to be strong. Savvannah, you have not only been my life coach for the past 4 years (more like forever), but my confidant. Thank you for the constant pep talks, for volunteering your time to listen to my presentations (over and over again!)

or proofread any reports despite having a limited scientific background. Thank you especially for believing in me the most! Marl, I physically wouldn't have been able to write this thesis without you. Thank you for the laptop, icloud and desktop space and for all your support. Grandad and Nanny, thank you so much for your continuous encouragement and for always reminding me that "I can do anything I put my mind to". Dad, thank you for allowing me to force you to train with me when I needed the frequent stress relief and for making me laugh out loud several times! Finally, to my nephews Marley and Zayne, thank you for the absolute joy and motivation you have given me. I hope one day you will be super proud of Aunty Sian!

I signed up to this journey not realising that you guys were automatically signing up too and I cannot thank you enough for sticking by me (and my tantrums) until the very end.

This thesis is wholehearted dedicated to My Family, especially my Angel (Shen'iyah x).

Abstract

The placenta is an important endocrine organ, secreting various placental-derived mediators which influence the maternal milieu to support the demands of pregnancy. A physiological metabolic consequence of pregnancy is a progressive increase in maternal insulin resistance mid-late gestation, thus facilitating a constant supply of glucose to the developing foetus. The insulin-secreting β-cells in the pancreatic islets of Langerhans undergo adaptations by enhancing insulin secretory responses to elevations in plasma glucose and increasing β-cell mass to compensate the insulin resistance. In some pregnancies, these adaptations fail and chronic hyperglycaemia and overt gestational diabetes mellitus (GDM) ensues. The corticotropin releasing hormone (CRH) system (including structurally related peptides, urocortin (UCN)1, -2 and -3 and the two cognate receptors, CRHR1 and CRHR2) is well known as the neuroendocrine system regulating the stress response as part of the hypothalamic-pituitary-adrenal (HPA) axis. However, extra-hypothalamic roles for this system in peripheral tissues have been reported, including within the endocrine pancreas. Increasing evidence also suggests that the CRH peptide family may be involved in peripheral metabolic control via direct actions on β-cells. Generally, circulating levels of CRH is low. However, we have previously reported that during mouse pregnancy, expression of CRH-like peptides increase in the placenta suggesting that these peptides may have pregnancy specific roles. Therefore, the aim of this project was to investigate whether there is a physiological role for the CRH system in the pancreatic islet adaptations to pregnancy.

Functional *in vitro* studies using isolated mouse islets and either static incubation or dynamic perifusion methodologies confirmed that activation of both CRHRs, using selective receptor agonists could enhance insulin release in response to elevations in glucose. Expression of CRH and all urocortin peptide mRNAs were confirmed in the mouse placenta along with both types of CRHR in mouse islets. Intriguingly, pregnant islets displayed a significant decrease in CRHR1 expression and maintained CRHR2 expression levels when compared to non-pregnant control islets. Moreover, mouse pregnancy is associated with a significant increase in circulating UCN2 levels with CRH, UCN1 and UCN3 levels unchanged.

Pharmacologically blocking endogenous CRHR signalling using selective and non-selective CRHR antagonists chronically administered via subcutaneously implanted osmotic minipumps *in vivo* during pregnancy, revealed a pregnancy–and receptor-specific phenotype in mice. Intraperitoneal glucose tolerance tests revealed a significant, albeit mild and transient, impairment to glucose tolerance with selective CRHR2 blockade but not with CRHR1 blockade which was associated with a decrease in glucose-stimulated plasma insulin levels, but not basal fasted insulin. No detectable effects on insulin sensitivity or the proliferative capacity of β-cells were observed.

Furthermore, no impairment to overall glucose homeostasis was observed with total (i.e. non-selective) CRHR blockade outside of pregnancy.

Modelling the beneficial effects of endogenous UCN2 during pregnancy by exogenously administering the peptide in an alternative animal model of impaired glucose homeostasis (i.e. the insulin resistant obese ob/ob mouse) highlighted the variable effect of UCN2 on glucose homeostasis between acute (i.p.) and chronic (osmotic minipump) administration. Overall, studies mimicking physiological pregnant levels of UCN2 revealed no significant alterations to glucose homeostasis indicating that the positive effects of UCN2 on β-cell function is confined to pregnancy and may suggest something unique about the pregnancy environment, perhaps synergy with other pregnancy mediators, is important for its functional benefit.

Preliminary clinical studies in pregnant women revealed a positive association between plasma UCN2 and insulin responses to oral glucose along with HOMA2-%β (index of β-cell function), consistent with the theory that higher levels of maternal UCN2 can directly enhance islet β-cell function and suggests a conserved mechanism between mouse and human pregnancy. However, no significant differences in plasma levels of UCN2, UCN3 or CRH between healthy women and women diagnosed with GDM were observed, in line with the milder phenotype displayed *in vivo* in mice with endogenous UCN2 blockade during pregnancy.

In summary, the studies presented in this thesis suggest that an endogenous ligand, most likely UCN2, signalling via CRHR2, contributes to maintaining maternal normoglycaemia during pregnancy. The current data suggests that UCN2 may support β-cell adaptations by amplifying the insulin secretory response to the metabolic demand. Data from the clinical cohort appear to support the pregnancy-specificity of this signal which may be conserved in mouse and human gestation. Therefore, UCN2/CRHR2 signalling represents a novel signal involved in the pancreatic islet adaptations to pregnancy.

Table of Contents

Figures

Tables

Abbreviations

$[Ca2+]_i$	Intracellualr calcium concentration
/	Per
&	And
%	Percent
®	Trademark
<	Less than
>	More than
5-HT	Serotonin
AC	Adenyl cyclase
ACOG	American College of Obstetricians and Gynaecologists
ACTH	Adrenocorticotropin hormone
ADP	Adenosine monophosphate
AH	Antalarmin hydrochloride
ANOVA	Analysis of variance
AP	Alkaline phosphatase
AS-30	Antisauvagine-30
AS160	Akt substrate of 160 kDa
ATP	Adenosine triphosphate
AUC	Area under the curve
AVP	Arginine vasopressin
AVV8	Adeno-associated virus type 8
BMI	Body mass index
B_o	Maximum binding
BrdU	5-bromo-2'-deoxyuridine
BSA	Bovine serum albumin
c-Met	Hepatocyte growth factor receptor
Ca^{2+}	Calcium ion
cAMP	cyclic adenosine monophosphate
cDNA	Complementary dioxyribonucleic acid
cm	Centimeter
CNS	Central nervous system
CO2	Carbon dioxide
cpm	Counts per minute
Cq	Quantification cycle
CREB	cAMP response element-binding protein
CRF	Corticotropin releasing factor
CRH	Corticotropin releasing hormone
CRH-BP	Corticotropin releasing hormone- binding protein

CRHR	Corticotropin releasing hormone receptor
CRHR1	Corticotropin releasing hormone receptor type 1
CRHR2	Corticotropin releasing hormone receptor type 2
Ct	Threshold cycle
DAG	Diacylglycerol
DNA	Dioxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
DPP-4	Dipeptidyl peptidase-4
ds DNA	Double stranded dioxyribonucleic acid
EC50	Concentration of a drug that gives half-maximal response
EDTA	Ethylenediaminetetraacetic acid
EdU	5-Ethynyl-2´-deoxyuridine
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERK	Extracellular Signal-Regulated Kinase
EWN	Edinger-Westphal nucleus
FBS	Fetal Bovine Serum
FFAR1	Free fatty acid receptor 1
g	G-force
G	G-protein / Guanine nucleotide- binding proteins
g	Gram
G&G	Gey and Gey buffer
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCGR	Glucagon receptor
GDM	Gestational diabetes mellitus
GDP	Guanine diphosphate
GI	Gastrointestinal
GIP	Glucose-dependent insulinotropic polypeptide
GIPR	Glucose-dependent insulinotropic polypeptide receptor
GLP-1	Glucagon -like peptide 1
GLP-1R	Glucagon -like peptide 1 receptor
GLUT	Glucose transporter protein
GPCR	G-protein coupled receptor
GPR54/ KISS1R	Kisspeptin receptor
GSIS	Glucose stimulated insulin secretion
GSK-3	Glycogen synthase kinase-3
GTP	Guanine triphosphate
GTT	Glucose tolerance test
GWAS	Genone wide association studies
h	Hour

h/r CRH	Human/rat corticotropin releasing hormone
HAPO	Hyperglcemia and Adverse Pregnancy Outcomes
HCl	Hydrochloric acid
HFD	High fat diet
HGF	Hepatocyte growth factor
HOMA	Homeostasis Model Assessment
HOMA2-%β	Homeostasis Model Assessment b-cell function
HOMA2-IR	Homeostasis Model Assessment insulin resistance
HPA axis	Hypothalamic-pituitary-adrenal axis
hPL	Human placental lactogen
HRP	Horseradish peroxidase
Htr2b	Serotonin receptor 5-hydroxytryptamine receptor-2b
Htr3b	Serotonin receptor 5-hydroxytryptamine receptor-3b
i.p.	Intraperitoneal
IADPSG	International Association of Diabetes and Pregnancy Study Group
IBD	Irritable bowel disease
IHC	Immunohistochemistry
IL-6	Interleukin 6
INS-1	Rat insulinoma cell line
IP_3	Inositol 1,4,5 -triphosphate
IR	Insulin receptor
IRS	Insulin receptor substrate
ITT	Insulin tolerance test
IU	International Units
JAK	Janus kinase
K^{+ATP}	ATP sensitive potassium channel
kDA	Kilodalton
kg	Kilogram
Ki67	proliferation protein
kir6.2	Potassium subnit of KATP channel
KO	Knockout
l	Litre
LADA	Latent autoimmune diabetes of adults
LGA	Large for gestational age
MAPK	Mitogen-activated protein kinase
MEM	Minimum Essential Media
mg	Miligram
min	Minute
min	Minute
MIN6	Mouse insulinoma 6 cell line

MIP	Mouse insulin promoter
ml	Mililitre
mm	Milimeter
mM (mmol/l)	Milimolar
MODY	Maturity onset diabetes of the young
mRNA	Messenger ribonucleic acid
n	Number of samples
NCS	Newborn Calf Serum
NEFA	Non-esterified fatty acids
ng	Nanogram
NGT	Normal glucose tolerance
NHS	National Health Service
NICE	National Institute for Health and Care Excellence
nm	Nanometer
nM	Nanomolar
ns	Non-significant
NSB	Non-specific binding
ob/ob	Leptin deficient obese mice
oCRF	Ovine corticotropin releasing hormone
OGTT	Oral glucose tolerance test
OMP	Osmotic minipump
p	p value
P/S	Penicillin/streptomycin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD.	Pregnancy day.
PDK	PIP3-dependent kinase
PEG	Polyethylene glycol
PH	Pleckstrin homology
PI3K	Phosphoinositide-3 kinase
PIP_2	Phosphatidylinositol 4,5 bisphosphate
PKA	Protein kinase A
PKB / Akt	Protein kinase B
PL	Placental lactogen
PLC	Phospholipase C
pM	Picomolar
PNPP	para-Nitrophenylphosphate
POMC	Pro-opiomelanocortin
PP	Pancreatic polypeptide
PRL	Prolactin

PRLR	Prolactin receptor
PVN	Paraventricular nucleus
qPCR	Quantitative polymerase chain reaction
r	Correlation co-efficient
rcf	Relative centrifugal force
RIA	Radioimmunoassay
RLT	Buffer used in RNA extraction
RM-ANOVA	Repeated measure -Analysis of variance
RNA	Ribonucleic acid
RPE	Buffer used in RNA extraction
rpm	Revolutions per minute
RPMI-1640	Roswell Park Memorial Institute -1640
RT	Reverse transcription
RT-qPCR	Real time- quantitative polymerase chain reaction
RW1	Buffer used in RNA extraction
SAT	Subcutaneous adipose tissue
sCRHR2α	Soluble corticotropin releasing hormone receptor 2 alpha
SD	Standard deviation
SEM	Standard error of the mean
SGA	Small for gestational age
STAT	Signal transducer and activator of transcription
SUR1	Sulphonylurea subunit of KATP receptor
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TBS	Tris buffered saline
TCA cycle	Tricarboxylic acid cycle
T_m	Melting temperature
TMB	3,3',5,5'-Tetramethylbenzidine
TNF-α	Tumor necros factor alpha
TPH1	Tryptophan hydroxylase 1
TPH2	Tryptophan hydroxylase 2
UCN1	Urocortin 1
UCN2	Urocortin 2
UCN3	Urocortin 3
UCNs	Urocortins
UK	United Kingdom
v/v	Volume per volume
VDCC	Voltage dependent calcium channel
VIP	Vasoactive intestinal polypeptide
vs	versus

WHO	World Health Organization
WT	Wild type
α	Alpha
β	Beta
δ	Delta
µg	Microgram
µl	Microlitre
µm	Micrometer
µM	Micromolar

Chapter 1

Chapter 1 General Introduction

1.1 Glucose homeostasis

Glucose is the major fuel substrate for normal functioning of cells within the body. It is essential to precisely regulate the concentration of glucose in the blood so physiological processes are performed optimally, whilst also preventing pathological states such as hyperglycaemia or hypoglycaemia (abnormally high or low blood sugar levels respectively). Normal glycaemia fluctuates throughout the day reflecting the pre-prandial (before meal ingestion) and post-prandial (after meal ingestion) nutritional status. In normal healthy individuals, fasted glucose levels are approximately in the range of 4 - 6 mmol/l. Mice typically show higher blood glucose levels than seen in humans, ranging between 7 – 9 mmol/l (King, 2012; Kowalski and Bruce, 2014). The continuous balance between glucose uptake, utilisation and storage versus glucose production, relies on mechanisms allowing for the detection in changes of circulating glucose and consequently the appropriate hormonal secretory response. The primary glucoregulatory hormones include glucagon and insulin. These hormones are secreted from the α- and β-endocrine cells of the pancreas respectively, an organ considered to be the "head-quarters" for glucose homeostasis.

Following nutrient absorption, circulating glucose levels rise which stimulates the release of insulin. Insulin is an anabolic hormone and promotes the uptake and storage of circulating glucose. Glucagon on the other hand, has an opposing action to that of insulin and is a catabolic hormone, preventing further decrements in blood glucose below the normal range, for example during fasting. It does this by stimulating the production and secretion of glucose via hepatic glycogenolysis (breakdown of glycogen stores) and hepatic and renal gluconeogenesis (production of endogenous glucose from non-carbohydrate precursors), thus maintaining euglycemia. The relative balance of these processes relies on specific glucose sensing mechanisms within pancreatic endocrine cells, transmitting fluctuations in extracellular glucose into a cascade of intracellular signals. Elevations in circulating glucose results in increased glucose uptake into the β-cell through glucose transporter proteins (GLUT) constitutively expressed on β-cell plasma membranes; GLUT1 in humans and GLUT2 in rodents (De Vos et al., 1995; Gould and Holman, 1993; van de Bunt and Gloyn, 2012). These low affinity, high capacity transporters facilitate the entry of glucose into the β-cell, allowing for rapid equilibration of intracellular glucose to that of the prevailing plasma concentration, triggering the subsequent phosphorylation of glucose by the enzyme, glucokinase. The consequent cascade of intracellular events concerning glucose metabolism via the glycolysis pathway produces an increase in adenosine triphosphate (ATP), which blocks ATP-dependent potassium channels (K^+_{ATP}) and causes depolarisation of the β-cell membrane. The resulting cell depolarisation allows influx of

Ca^{2+} into the cell which triggers the secretion of insulin-containing granules from the β-cell (Wilcox, 2005). Therefore, glucose is considered the most potent stimulator of insulin release and thus this regulated response is commonly referred to as "glucose-stimulated insulin secretion" (GSIS) (Komatsu et al., 2013; Pia V Röder et al., 2016).

Insulin acts on target insulin-sensitive tissues such as skeletal muscle, adipose tissue and liver hepatocytes via interaction with its cognate tyrosine kinase receptor, the insulin receptor (IR), to accelerate glucose uptake and/or metabolism. In skeletal muscle and adipose tissue, glucose entry involves the recruitment of the insulin regulated glucose transporter, GLUT4. In the unstimulated state (i.e. in the absence of insulin), GLUT4 is concentrated within storage compartments within the cell. Upon insulin signalling, GLUT4 translocates from these compartments to the cell surface where it transports glucose from the extracellular milieu into the cell. Conversely, the predominant glucose transporter isoform highly expressed in hepatocytes (the liver being a major site for excess glucose storage) is GLUT2 which is always present on the cell membrane. Glucose transport into the liver is not dependent on insulin. However, once glucose is transported inside the hepatocytes, insulin stimulates glycogen synthesis by inducing transcription of key glycogenic proteins. This includes activation of the enzyme hexokinase (enzyme phosphorylates glucose) and glycogen synthase (enzyme catalyses production of glycogen) (Akpan et al., 1974; Petersen and Shulman, 2018). Insulin also exerts an inhibitory action on enzymes involved in glucose production by the liver (Hatting et al., 2018). Despite these tissue specific processes, the significance and implications of both GLUT4 and GLUT2 in normal glucose homeostasis and elements of insulin resistance have been displayed in heterozygous (GLUT4+/-) or homozygous (GLUT2-/-) knock out mouse models. Both models exhibit glucose intolerance and characteristics of diabetes (Guillam et al., 1997; Stenbit et al., 1997).

Whilst the net effect of insulin signalling is to lower glucose excursions, glucagon signalling via its stimulatory heterotrimeric G-protein coupled receptor (GPCR) (GCGR), predominantly expressed in the liver, has a hyperglycaemic effect. Although glucose and the insulin response is well understood, there is still much to be elucidated about the metabolic modulation of glucagon signalling. Under physiological conditions, paracrine signalling between the pancreatic β- and δ-cells with α-cells, as well as glucose directly, act to module the level of glucagon secretion (Zhang et al., 2013).

1.1.1 The islets of Langerhans

The pancreas is primarily responsible for two physiological secretory functions; 1) macro-nutrient digestion via the secretion of various digestive enzymes such as pancreatic lipase into the small intestine via the pancreatic duct and 2) energy homeostasis via the secretion of various hormones

such as insulin, secreted directly into the bloodstream. These functions represent the exocrine and endocrine portions of the organ respectively. The majority of the pancreas is composed of acinar or exocrine tissue and the minority represents the endocrine portion (accounting for 1- 2% of total pancreatic volume) identified by "island-like" cell clusters dispersed throughout the pancreas. These are referred to as the islets of Langerhans (Pia V Röder et al., 2016). It is estimated that the adult human pancreas contains between 3.8 - 14.2 million islets and between 1,000 – 5,000 in mice. However, the distribution of islet size is similar between both species (100 – 200 μm in diameter) (Da Silva Xavier, 2018; Dolenšek et al., 2015; Steiner et al., 2010). This inter-species conservation in islet size probably reflects the crucial islet diameter required for optimal islet function and intra-islet communications, especially as islets are multicellular, consisting of 5 different endocrine hormone-secreting cell types. Although, islet size is fairly constant between humans and mice, there are distinct differences in the islet cell ratios and anatomical arrangement. Insulin secreting β-cells are the predominant endocrine cell type in both rodent and human islets although human islets contain proportionally fewer β-cells (70- 80% in rodents versus 50- 60% in humans). Glucagon secreting α cells constitute approximately 15 - 20% and 30 – 40% in rodents and humans respectively. Somatostatin secreting δ-cells are fewer than 10% of the islet cell population in both species. The remaining pool of endocrine cells are composed of PP-cells, and ε-cells, secreting pancreatic polypeptide and ghrelin, respectively (Cabrera et al., 2006). The spatial organisation of these cell types are thought to have implications for overall islet function, with more heterologous contact between cell types in human islets characterised by a random, "scattered" cyto-architecture whereas the dense β-cell core surrounded by a mantle of non β-cells, is thought to represent the more homologous β-β cell contacts in rodent islets (see Figure 1-1) (Bosco et al., 2010; Cabrera et al., 2006). Not only does the morphology between human and rodent islets differ slightly but it has also recently been reported that differences in the intracellular metabolic pathways and enzymes involved in coupling responses to insulin release differ between the species (MacDonald et al., 2011). Thus, it is important to note these considerations when translating findings from studies of mouse islet physiology to humans.

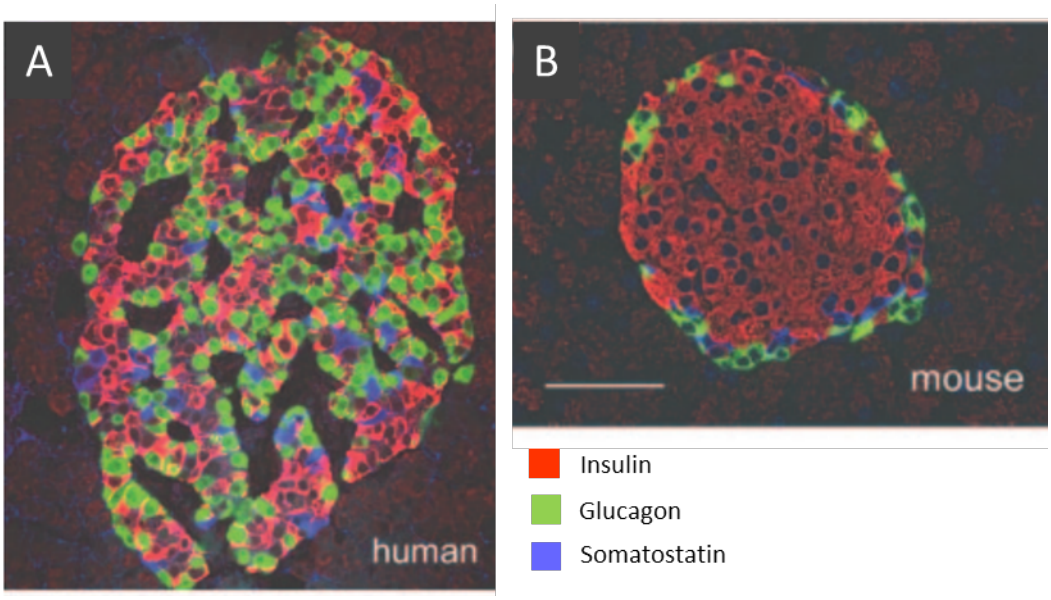

Figure 1-1 Immunofluorescent images of pancreatic sections containing human (A) and mouse (B) islets of Langerhans. Immunoreactive insulin-secreting β-cells (red), glucagon-secreting α-cells (green) and somatostatin -secreting δ-cells (purple), are shown to illustrate the distinct distribution of endocrine cell types between human and mouse islets. Human islets exhibit a random distribution of endocrine cells whereas mouse islets reveal a core of insulin secreting β-cells, surrounded by a mantle of fewer α-and δ-cells. Image taken from (Cabrera et al., 2006).

Islets are highly vascularised receiving approximately 15% of total pancreatic blood supply. Despite the differences in cellular distribution, most cells within the islet (86% of human islet α-cells and 77% of β-cells), are closely associated to vascular endothelial and smooth muscle immunoreactive cells (Cabrera et al., 2006). In addition to the islet microcirculation, the presence of gap junctions between islet cells, creating inter-cellular spaces of approximately 15-20 nm, means chemical signalling can rapidly and directly influence neighbouring islet target cells (Orci et al., 1975). The microanatomy of islets also reveals neural control, with pancreatic islets innervated by both parasympathetic and sympathetic nerve terminals (Thorens, 2014). However, it is also recognised that neurotransmitters and neuropeptides derived from islet cells themselves, also participate in the autocrine and paracrine signalling within islets and therefore can have positive or negative effects on hormone release. Thus, islets are aptly equipped to rapidly sense fluctuations in circulating glucose, respond to external cues, as well as deliver endocrine signals to maintain glucose homeostasis. Optimal glucose regulation therefore involves a sophisticated intra-islet network integrated with a number of organ/tissue - islet axes which can also influence islet behaviour. The integration of these signals represents the complex and dynamic dialogue that is the crosstalk between pancreatic islets and the rest of the body.

1.1.2 The regulation of insulin secretion

The regulation of insulin secretion involves a cascade of intracellular signalling pathways, that either govern the triggering/initiation or amplification of hormone secretion. The triggering response of β-cell insulin secretion is well established, with elevations in circulating glucose (or nutrient intake) being the primary stimuli. This response is therefore termed glucose-stimulated insulin secretion (GSIS). The amplifying pathways ensures optimisation of insulin secretion only once the glucose dependent pathway has been initiated and primarily employs distinct β-cell surface receptors. A hallmark of the insulin secretory response is the biphasic secretion profile in response to elevated glucose. This consists of a rapid but transient first phase, followed by a prolonged and maintained second phase (Curry et al., 1968; Pia V Röder et al., 2016). Disturbances in insulin regulation can cause hypoglycaemia (in the case of excessive insulin secretion) or diabetes when insufficient insulin secretion occurs.

1.1.2.1 Initiation pathway of insulin secretion

Generally, glucose metabolism in β-cells is essential to induce insulin secretion. However, other nutrients including non-esterified fatty acids (NEFA) and small amino acids can also stimulate an insulin secretory response (Newsholme et al., 2006; Newsholme and Krause, 2012). A rise in circulating glucose permits its intracellular uptake in β-cells via glucose transporters, predominantly GLUT2 in rodent β-cells and GLUT1/3 in human β-cells (McCulloch et al., 2011) by facilitated diffusion. Subsequent phosphorylation of glucose to glucose-6-phosphate and entry to the glycolysis pathway is catalysed by the enzyme glucokinase. The kinetics of glucokinase (i.e. high K_m) means that this reaction serves as the rate-limiting step in glucose metabolism and therefore ensures insulin release is proportional to the prevailing circulating glucose concentration. Mitochondrial metabolism of pyruvate (the end product of glycolysis) via the tricarboxylic acid (TCA) cycle, generates adenosine triphosphate (ATP), which is pivotal in coupling glucose metabolism to insulin secretion. The concomitant increase in the cytosolic ATP/ADP ratio enables binding of ATP to ATP sensitive potassium (K^+_{ATP}) channels in the plasma membrane of the β-cell. These channels are hetero-octamers consisting of four potassium subunits (kir6.2), forming the pore through which potassium ions flow, surrounded by four sulfonylurea receptor subunits (SUR1) which have a regulatory role (Ashcroft and Gribble, 1998; Shyng and Nichols, 1997). Under euglycaemic conditions, the resting membrane potential is maintained by efflux of potassium ions. Binding of ATP to the kir6.2 subunits causes channel closure and subsequent reduction in potassium efflux. This induces β-cell membrane depolarisation (Cook and Hales, 1984). The electrophysiologic capacity and structure of the K^+_{ATP} channels renders them sensitive to agents such as sulfonylureas and diazoxide which can close or open the channel respectively, and thus influence overall insulin secretion by bypassing glucose

metabolism. Hence, the therapeutic potential of sulfonylureas such as glibenclamide or glipizide have been exploited in the management of type 2 diabetes mellitus (T2DM) (Rendell, 2004).

Membrane depolarisation promotes the opening of voltage dependent Ca^{2+} channels (VDCCs), an influx of Ca^{2+} ions down a concentration gradient and therefore leads to an increase in intracellular calcium concentrations ($[Ca^{2+}]_i$). Ca^{2+}- dependent activation of exocytotic machinery follows, triggering the fusion of insulin containing granules with the plasma membrane, allowing for release of their content into the circulation (Eliasson et al., 1996; Gillis and Misler, 1992; Proks and Ashcroft, 1995) (detailed in Figure 1-2). Cytosolic Ca^{2+} also serves as a self-amplifier by inducing the activation of β-cell phospholipase C (PLC) leading to the generation of the second messengers, inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 can rapidly mobilise intracellular calcium from endoplasmic reticulum (ER) stores thus further contributing to $[Ca^{2+}]_i$. This also highlights a point of convergence between the nutrient-induced secretory pathway and one which can be employed by other non-nutrient insulin secretagogues.

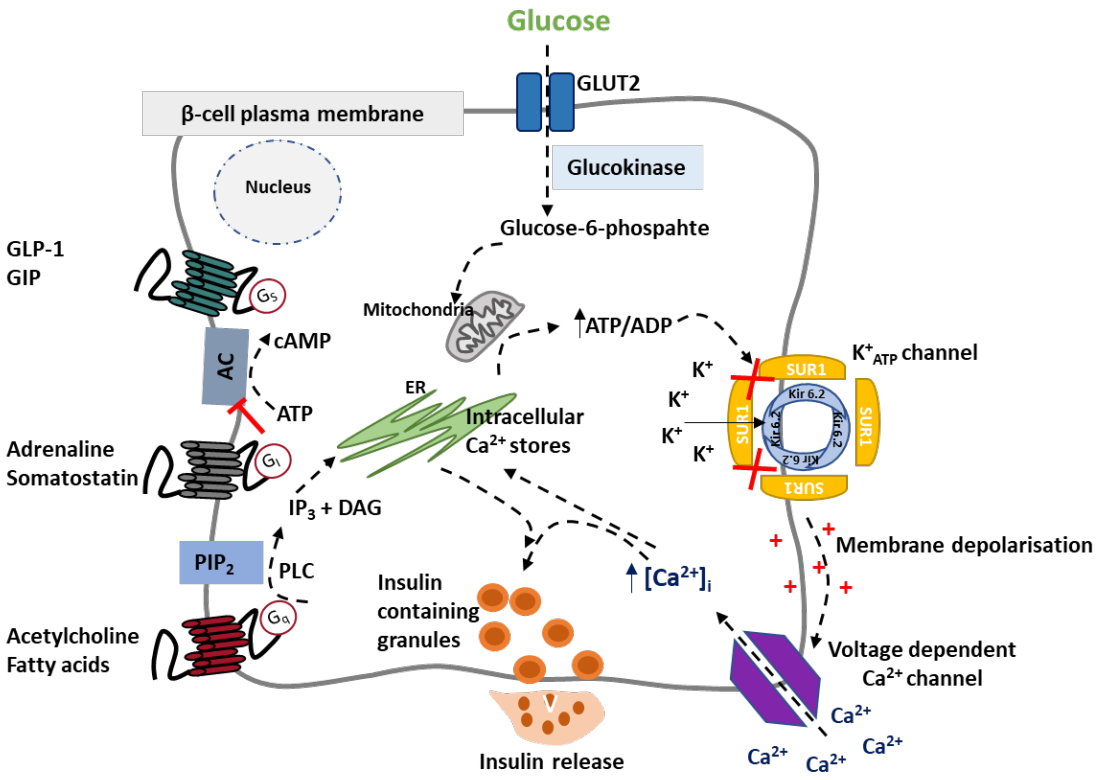

Figure 1-2 Schematic representation of the regulation of insulin secretion from pancreatic β-cells. Increases in circulating glucose is transported into β-cells via plasma membrane glucose transporters (GLUT2). Entry of glucose into the cell and subsequent metabolism, results in an increase in the ATP/ADP ratio, sequentially leading to the closure of K^{+}_{ATP} channels. Channel closure prevents K^{+} efflux, causing membrane depolarisation and stimulates the opening of voltage-dependent Ca^{2+} channels. Influx of Ca^{2+} eventually leads to the exocytosis of insulin-containing granules. β-cells also contain plasma membrane G-protein coupled receptors (GPCRs) that are able to module the extent of insulin release via specific intracellular signalling pathways upon ligand binding.

1.1.2.2 Amplification pathways of insulin secretion

β-cells also have the innate capacity to respond to K^{+}_{ATP} channel -independent stimuli to augment GSIS by either enhancing or abrogating secretion. These pathways often involve the more commonly expressed, G-protein coupled receptors (GPCRs), on β-cell plasma membranes. Although the human β-cell expresses approximately 300 different GPCRs (Amisten et al., 2013), there are a limited number of intracellular effectors systems employed following ligand binding and receptor activation. Neurotransmitters (e.g. acetylcholine), neuropeptides (e.g. Vasoactive intestinal polypeptide (VIP)) and various biological hormones (e.g. Glucagon like peptide (GLP-

1)) are examples of non-nutrient stimuli that can potentiate insulin secretion via their cognate β-cell receptors (Cairano et al., 2016).

All GPCRs share a similar structure; a single polypeptide consisting of an extracellular N-terminal domain (for ligand binding) linked via seven transmembrane spanning domains to the intracellular C- terminus (for signal transduction). Ligand-receptor binding causes a conformational change in the receptor allowing the interaction of the C-terminus with heterotrimeric guanine nucleotide binding proteins (G-proteins) (Rosenbaum et al., 2009). These G-proteins form a complex composed of 3 subunits; α, β and γ, either bound by guanine diphosphate (GDP) in the inactive state or guanine triphosphate (GTP) in the activate state. The α-subunit of the complex directly interacts with GTP or GDP, permitting the regulation of target proteins such as enzymes or ion channels that can influence second messenger pathways within the cell (see Figure 1-3). Four main types of α-subunit have been reported: $G\alpha_s$, $G\alpha_q$, $G\alpha_i$ and $G\alpha_{12/13}$, each displaying distinct downstream signalling responses depending on the receptor and cell type they are expressed (Gilman, 1987; Rosenbaum et al., 2009). Generally, receptor coupling to $G\alpha_s$ or $G\alpha_q$ increases insulin secretion whereas activation of the $G\alpha_i$ sub-unit inhibits insulin secretion. The consequence of $G\alpha_{12/13}$ coupling on insulin secretion is still unclear (Ahrén, 2009; Winzell and Ahrén, 2007). The effector proteins for $G\alpha_s$ and $G\alpha_q$ are the membrane bound enzymes, adenylyl cyclase and phospholipase C (PLC), respectively. Adenylyl cyclase catalyses the conversion of ATP to cyclic adenosine monophosphate (cAMP) whereas PLC stimulates the production of second messengers, inositol 1,4,5 trisphosphate (IP_3) and diacylglycerol (DAG) from membrane phosphatidylinositol 4,5 bisphosphate (PIP_2). Downstream signalling ultimately results in the activation of kinases, PKA in the case of $G\alpha_s$ and PKC for $G\alpha_q$, which can facilitate a rise in intracellular Ca^{2+} and thus amplify the insulinotropic effect of glucose (see Figure 1-3).

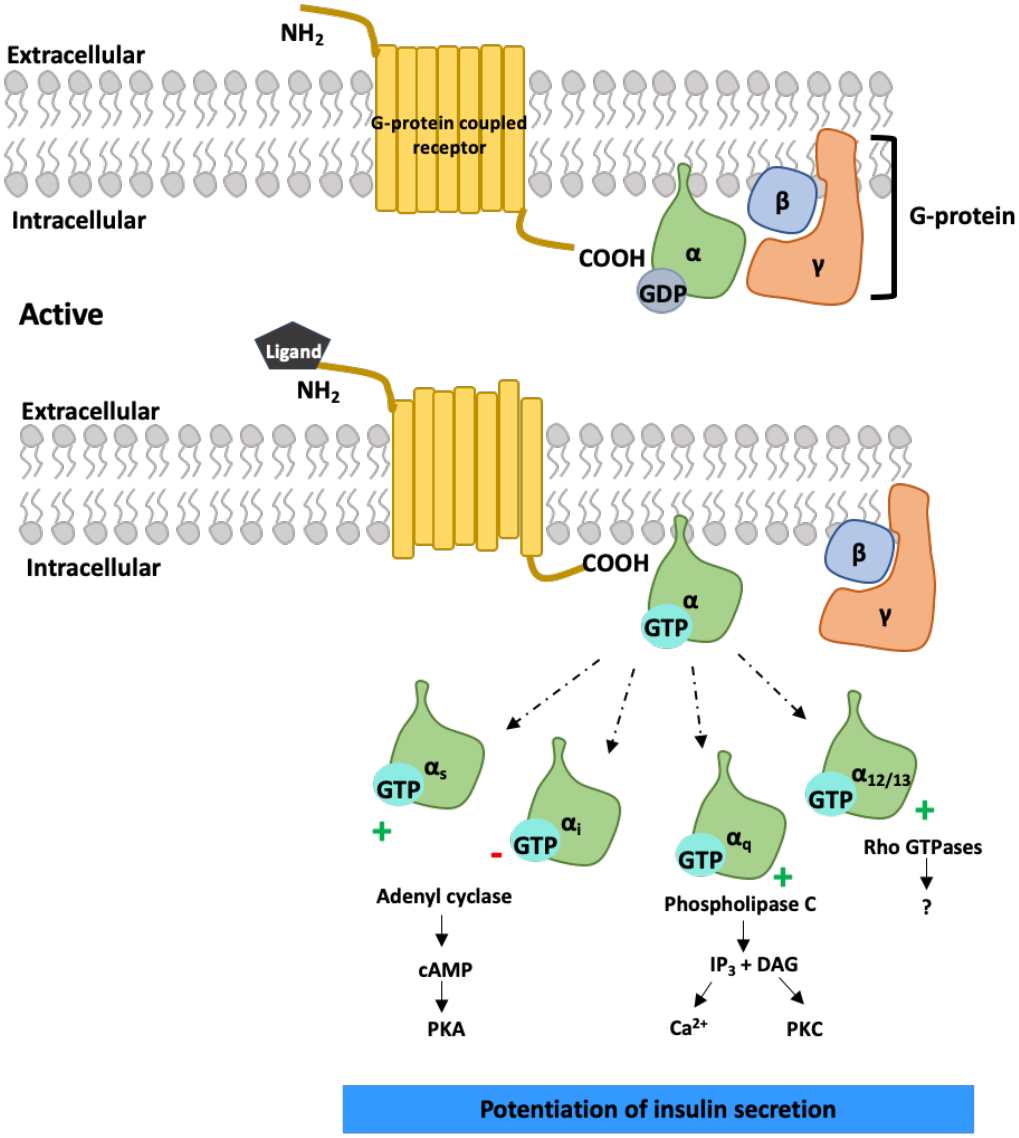

Figure 1-3: Schematic of G-protein coupled receptor activation and signalling. In the inactive state, the transmembrane GPCR interacts with the intracellular G-protein (all subunits associated) which is bound by GDP. Receptor activation (as a result of ligand binding) causes a conformational change in the receptor which triggers the dissociation of the Gα subunit of the G-protein from the β and γ subunit and GTP is exchanged for the bound GDP. Gα activation can then activate (or inhibit) target effector proteins, producing specific intracellular second messenger molecules that can regulate pathways involved in insulin secretion.

Due to the modulatory effect of islet GPCRs/ligands on β-cell function and expansion, they have proven to be attractive targets for anti-diabetic therapies, particularly for T2DM. Therefore, GPCR agonists which can enhance endogenous insulin production and/or stimulate β-cell proliferation are frequently undergoing development and clinical trials. One of the major successful GPCR based therapies includes GLP-1R agonists/ mimetics. GLP-1 analogues such as

Exenatide, act directly at β-cell GLP-1Rs to have the same effect as endogenous incretin peptides (i.e. increase insulin and inhibit glucagon secretion along with improved β-cell survival) but with prolonged duration of action (Oh and Olefsky, 2016). Other potential islet GPCR targets include free fatty acid receptor 1 (FFAR1) (e.g. GPR40) however, agonists targeting this receptor have been largely unsuccessful because of adverse effects or toxicity issues (Ahrén, 2009; Persaud, 2017). Despite GPCRs representing tractable targets there are still only a limited number of drugs approved for diabetes therapies. As human islets express 293 GPCRs (Amisten et al., 2013) there is plenty of scope to investigate the functional role and utility of novel islet GPCR ligands that may display favourable effects on β-cell physiology. Anti-diabetic interventions targeting islet GPCRs appear to be one of the most promising pharmacological approaches attempting to reduce the growing burden of diabetes worldwide.

1.2 Diabetes Mellitus- The growing crisis

Diabetes is a chronic metabolic disease characterised by elevated glucose levels also referred to as hyperglycaemia. In just under four decades the number of people reported to be living with diabetes has quadrupled to over 400 million people worldwide (Roglic and World Health Organization, 2016) a figure which is expected to rise to 629 million by 2045 (Cho et al., 2018). In the United Kingdom alone 4.7 million people are living with the disease with a diagnosis being made every 2-3 minutes (Diabetes UK, 2019). The quality of life for those living with diabetes is greatly reduced especially when the disease is poorly managed. Individuals have an increased risk of developing secondary complications affecting the vasculature of the heart (termed macrovascular complications), eyes, kidneys, nerves and lower limbs (referred to as microvascular complications). These can be life-threatening and subsequently contribute to the premature mortalities of patients each year (approximately 1.6 million deaths in 2016 according to the World Health Organisation (WHO)). The diabetes epidemic has an estimated global expenditure of USD 850 billion (as of 2017) (Cho et al., 2018), with an estimated annual expenditure to the National Health Service (NHS) of at least £10 billion (Diabetes UK, 2019). Approximately 80% of this expenditure is consumed by the associated complications of diabetes, including inpatient care due to potential kidney and/or cardiovascular complications. Diabetes prevalence is increasing at an alarming rate, yet it is estimated that a high proportion (just under 50%) of individuals living with the disease are undiagnosed. The impact to people's lives and the financial cost to economies is therefore anticipated to escalate. Thus, diabetes is considered a major global health challenge and international efforts to improve health outcomes along with developing preventative strategies are now underway in order to attenuate the growing crisis.

1.3 The spectrum of Diabetes

Decades of research has now led to a better understanding of diabetes as a complex spectrum of metabolic disorders characterised by chronic hyperglycaemia. The distinction between the various diabetic phenotypes, which was historically forged depending on age of onset and insulin dependency (Zaccardi et al., 2016), is now reflective of the clinical manifestations of the disease upon diagnosis. Broadly speaking, diabetes is classified into three categories; Type 1 (formerly known as insulin-dependent), Type 2 (formerly classified as non-insulin dependent) and Gestational diabetes mellitus (GDM) (ADA, 2019). Type 1 diabetes mellitus (T1DM) is well recognised as an autoimmune disorder caused by destruction of insulin producing cells (β-cells) and usually results in absolute insulin deficiency (Eisenbarth, 1986; Zaccardi et al., 2016) (insulin being the only hormone that permits the storage of fuel during the absorptive phase). Varying degrees of reduced target tissue insulin responsiveness (i.e. insulin resistance) and relative defects in insulin secretion are characteristic of both patients with Type 2 diabetes mellitus (T2DM) or GDM, although patients diagnosed with diabetes during pregnancy (in the case of GDM) are distinct from pregnant women with pre-existing diabetes despite sharing overlapping characteristics. Other less common forms of diabetes which do not entirely fall into these categories are referred to as "specific types" of diabetes such as monogenic diabetes syndromes, e.g. maturity-onset diabetes of the young (MODY) and neonatal diabetes. Other rare forms of diabetes include Latent autoimmune diabetes of adults (LADA) and drug/chemical - induced diabetes.

Of all diabetes cases reported in the UK, T1DM accounts for approximately 8% and T2DM accounts for approximately 90%. The remaining 2% represent those individuals with the rare/specific types of diabetes (Diabetes UK, 2019). These figures do not include those with gestational diabetes as typically, the hyperglycaemia resolves after giving birth and is temporary in most cases. Nonetheless, up to 10% of all pregnancies are complicated by this type of diabetes (Gilmartin et al., 2008). It is now recognised that women previously diagnosed with GDM have a substantially increased risk (~7 times) of developing future T2DM compared to a normoglycemic pregnancy (Bellamy et al., 2009a; Zhu and Zhang, 2016), thus contributing to the overall increased prevalence of Type 2 diabetes in particular.

1.3.1 Type 2 diabetes mellitus (T2DM)

T2DM is a multifactorial diabetic syndrome with genetic and environmental influences contributing to defects in both insulin secretion and action. The hallmark characteristics of T2DM is tissue (mainly liver and skeletal muscle) insulin resistance, impaired regulation of hepatic glucose production and β-cell dysfunction (Mahler and Adler, 1999). Although the sequence and

relative importance of each pathological mechanism in disease onset and progression is still debated, it is clear that early disruptions in the crosstalk between the pancreas and insulin target tissues can manifest into overt hyperglycaemia and thus T2DM.

Progressive insulin resistance has long been recognised as a critical determinant for T2DM. The diminished response to insulin, however, is usually initially compensated by β-cell insulin hypersecretion (hyperinsulinemia) (Zaccardi et al., 2016). Progression to diabetes is believed to occur when β-cells fail to compensate adequately for insulin resistance by secreting enough insulin. Whether hyperinsulinemia is simply a compensatory response to insulin resistance has been challenged by observations that hyperinsulinemia appears to precede decreases in insulin sensitivity (Erion and Corkey, 2018; Weyer et al., 2000). Increased basal insulin may therefore be directly contributing to and/or sustaining the insulin resistance in T2DM (Shanik et al., 2008). As a result, a vicious cycle of hyperglycaemia, hyperinsulinemia and insulin resistance ensues.

Given the substantially increased risk of developing T2DM in those with a family history of diabetes, it is unsurprising that individual genetic predisposition is associated with the disease. Genome wide association studies (GWAS) have enabled considerable progress in the discovery of genetic risk factors for T2DM. Over 70 susceptibility loci have been associated with T2DM (Sun et al., 2014) with most of the susceptibility genes affecting β-cell function, including *TCF7L2*, *KCNJ11* and *SLC30A8* (Park, 2011). A number of lifestyle factors including physical inactivity, excess alcohol intake and cigarette smoking have also been linked to the development of T2DM (Chen et al., 2012). Still, the strongest risk factor for T2DM development is obesity (Hu et al., 2001; Nolan et al., 2011). Of those diagnosed with T2DM, approximately 90% are overweight or obese (Public Health England, 2014) and the surge in T2DM prevalence is believed to parallel the obesity epidemic over the past decade. Obesity is frequently associated with systemic low-grade inflammation which may promote adipose tissue dysfunction (van Greevenbroek et al., 2013). As such, higher levels of circulating proinflammatory cytokines and alterations in the secretory profile of adipose-derived hormones (adipokines) (e.g. tumour necrosis factor alpha (TNFα), leptin and adiponectin) may interfere with normal insulin signalling (Scheen, 2003). Therefore, the obesogenic environment may contribute to the pathogenesis of T2DM.

The pathophysiology of T2DM is accompanied by additional hormone dysregulation besides those associated with the adipose tissue and β-cell. Disturbances to islet α-cell hormone (i.e. glucagon) secretion and gut incretin hormone (such as Glucagon-like peptide 1 (GLP-1)) secretion have also been reported in Type 2 diabetic patients (Baron et al., 1987; Dunning and Gerich, 2007; Toft-Nielsen et al., 2001). Hyperglucagonemia (excessive glucagon secretion) is commonly present in patients with T2DM, despite basal hyperglycaemia (Baron et al., 1987;

Dunning and Gerich, 2007). Impaired suppression of glucagon secretion exacerbates the increase in plasma glucose levels by stimulating hepatic glucose production, however the underlying cause of α-cell dysfunction is still elusive.

GLP-1 is a gut hormone secreted by enteroendocrine cells into the blood following meal ingestion. GLP-1 signals to the brain to induce satiety as well as to the gut to delay gastric emptying. Additionally, GLP-1 potentiates insulin secretion by activating its cognate GLP-1 receptor (GLP-1R) present on islet β-cells in response to a rise in circulating glucose, termed the incretin effect (Ahrén, 2009). α-cell glucagon secretion is also inhibited by GLP-1 (MacDonald et al., 2002). A diminished incretin effect has been observed in T2DM, with patients displaying significant reductions in GLP-1 secretion compared to normal glucose tolerant individuals (Toft-Nielsen et al., 2001). However, the loss of incretin activity in T2DM is most likely a consequence of the diabetic state as opposed to a primary factor in the pathogenesis. Importantly, the heterogenous rate of progression of individuals from impaired glucose tolerance to T2DM highlights the complexity in the underlying mechanisms and clinical manifestations of the metabolic syndrome with varying degrees of insulin resistance, β-cell dysfunction, hyperglucagonemia and adipokine dysregulation playing a role (Mahler and Adler, 1999; Nolan et al., 2011).

1.3.2 Distinction between T2DM and GDM

Gestational diabetes mellitus (GDM) is a common complication of pregnancy resulting in glucose intolerance and hyperglycaemia that is unique to pregnancy. Similar to T2DM, reduced insulin sensitivity and impaired insulin secretion are key pathophysiologic features of GDM (Johns et al., 2018a). However, the dysglycemia present in GDM is generally transitory and glucose tolerance normalises post-pregnancy. Therefore, GDM is considered a distinct form of diabetes and hence has separate diagnostic criteria to that of T2DM.

However, given the considerable aetiological over-lap between both conditions, including similar risk factors for disease development and pathophysiological mechanisms, the dogma that a possible common origin for GDM and T2DM exists has been debated (Pendergrass et al., 1995; Zajdenverg et al., 2017). This argument is further supported by the markedly increased risk of women with previous diagnosis of GDM developing future T2DM compared with those who had a normoglycemic pregnancy (Bellamy et al., 2009b). As such, GDM is now classified as a risk factor for subsequent T2DM development. Whether or not GDM represents an early stage in the natural progression of T2DM still requires further investigation. It is also unclear whether women who develop GDM have an underlying genetic predisposition affecting the ability of their islets to cope with metabolic stress and thus, whether the pregnancy environment actually unveils this pre-existing metabolic abnormality. Research efforts investigating the possible link between both

conditions may therefore not only be of importance in understanding more about the pathophysiology of GDM and T2DM but may also identify potential windows of opportunity to therapeutically intervene in disease progression. As GDM is the principle subtype of diabetes associated with this thesis, more discussion on glucose homeostasis during pregnancy and the pathophysiology of GDM will be presented below.

1.4 Gestational diabetes

Gestational diabetes mellitus (GDM) has historically been defined as glucose intolerance with onset or first recognition during pregnancy (Metzger, 1991). Over the years, this definition has been revised in attempt to provide greater clarity and more accurately stratify patient diagnosis. Therefore, the present definition describes gestational diabetes as any degree of glucose intolerance diagnosed in the second or third trimester of pregnancy, that is not clearly overt diabetes (Association, 2019) and thus, does not include women who have undiagnosed Type 2 and more uncommonly, Type 1 diabetes. The last two decades have seen a rise in the prevalence of gestational diabetes, affecting 7-10% of pregnancies worldwide (Behboudi-Gandevani et al., 2019). Prevalence figures can rise significantly in women from ethnic minority groups, particularly in Southeast Asian populations (Dickens and Thomas, 2019; Dornhorst et al., 1992). Therefore, estimates can vary widely depending on the underlying risk factors of the population studied as well as the diagnostic criteria applied.

GDM onset is associated with several risk factors, including advancing maternal age, increase in obesity or pre-pregnancy body mass index (BMI) and increased pregnancy weight gain. High-risk individuals also include those with a family history (first degree relatives) of diabetes, previous history of GDM and women of Asian, Hispanic or Black Caribbean ethnicities (Dickens and Thomas, 2019). A number of genetic risk factors have also been implicated in predisposing women to GDM including polymorphisms in *TCF7L2 rs7903146* and *CDKAL1 rs7756992* which are also risk alleles for Type 2 diabetes (Lauenborg et al., 2009). Given the similarities between GDM and Type 2 diabetes with respect to susceptibility genes, obesogenic influence and the significant risk (~ 50- 60%) of women with GDM to subsequently develop Type 2 diabetes in later life, it has been suggested that both diseases share a common pathogenic pathway. Whilst GDM shares a similar pathological profile to T2DM a comprehensive understanding of the GDM-specific pathogenic mechanisms is still lacking. Like T2DM, GDM is characterised by pancreatic β-cell dysfunction on a background of chronic insulin resistance (Plows et al., 2018) but the pregnancy specific physiology will be discussed later in this introduction.

1.4.1 Screening and diagnosis of GDM

Unfortunately, an international consensus regarding the timing of screening and diagnostic criteria/ glycaemic threshold used to make a firm diagnosis of the condition is lacking. Whether universal screening (i.e. all pregnant women undergo diagnostic testing) or risk-based screening (only those women with the risks discussed above undergo diagnostic testing) is employed, is also under debate. Universal screening has however, been reported to be a superior approach than risk-based screening and although more cases are diagnosed, these pregnancies are associated with improved outcomes (Griffin et al., 2000).

Generally speaking, the diagnostic criteria for GDM falls into two types of approaches; one-step screening versus two-step screening. The one-step screening criteria has been largely influenced by the landmark Hyperglcaemia and Adverse Pregnancy Outcomes (HAPO) study, which revealed a continuous association between maternal blood glucose levels and adverse maternal and fetal outcomes (HAPO Study Cooperative Research Group et al., 2008). Researchers used a 75-g oral glucose tolerance test (OGTT) at 24-32 weeks to calculate the adjusted odds ratio for adverse pregnancy outcomes associated with an increase in fasting plasma glucose, 1-hour plasma glucose and 2-hour plasma glucose levels. Based on this study, the International Association of Diabetes and Pregnancy Study Group (IADPSG) recommend a one-step 75-g 2-hour OGTT for screening at 24-28 weeks' gestation with thresholds for diagnosis of GDM as follows; fasting plasma glucose ≥ 5.1 mmol/l, 1-hour plasma glucose ≥10.0 mmol/l or 2-hour plasma glucose ≥8.5 mmol/l, with only one abnormal reading required for diagnosis (Consensus Panel, 2010). A consequence of the study was that these diagnostic thresholds reflected lower thresholds than those that were currently applied for diagnosis of overt diabetes. This was due to there being no clear maternal glycaemic threshold at which risks increased. However, critics of the lower threshold criteria have argued that this leads to over diagnosis of GDM (Behboudi-Gandevani et al., 2019).

The two-step screening approach is suggested to address the increased rate of potential false positive diagnoses that may occur with one-step screening and is endorsed by the American College of Obstetricians and Gynaecologists (ACOG). This protocol recommends that high risk women should be screened between 24-28 weeks' gestation with an initial 1- hour 50-g oral glucose challenge. If serum glucose values are positive, i.e. ≥ 7.2 - 7.8 mmol/l, it should be followed by a 3-hour 100-g oral glucose tolerance test to diagnose GDM. At least two abnormal plasma glucose readings from the following; fasting plasma glucose ≥ 5.3 mmol/l, 1-hour plasma glucose ≥10.0 mmol/l, 2-hour plasma glucose ≥8.6 mmol/l or 3-hour plasma glucose ≥ 7.8 mmol/l, are required for a diagnosis to be made (Harper et al., 2016; Johns et al., 2018b).

1.4.2 Adverse pregnancy outcomes associated with GDM

If hyperglycaemia during pregnancy is left unmanaged/ poorly controlled, this poses both short- and long-term health risks for mother and child. The short-term complications largely surround those associated with adverse maternal and fetal health during gestation as well as during and immediately after the labour period such as potential birth trauma. Mothers with GDM have a higher frequency of pre-term birth (9.4%), pre-eclampsia (9.1%) and primary caesarean delivery (24.4%) in comparison to non -GDM mothers (Buchanan et al., 2012). Macrosomia (larger than average new-born), shoulder dystocia (obstruction of fetal shoulder behind mother's pubic bone) and neonatal hypoglycaemia (resulting from fetal hyperinsulinemia in response to maternal hyperglycaemia) are among the most frequent adverse consequences for babies born to women with GDM (Reece, 2010). There is now a growing body of evidence to suggest that GDM and intrauterine exposure to GDM is associated with a higher risk of metabolic complications later in life for both mother and offspring. Progression to T2DM is 7-fold more likely in women with prior GDM compared to normoglycemic pregnancies, with the risk increasing markedly in the first 5 years following the indexed pregnancy (Bellamy et al., 2009b; Kim et al., 2002). Additionally, the likelihood of recurrent GDM in subsequent pregnancies is increased among women with previous GDM in comparison to women without GDM (Getahun et al., 2010). More recently, it has been reported that GDM predisposes mothers to future postpartum risk of cardiovascular disease (Kramer et al., 2019), although whether this risk is independent of subsequent T2DM remains inconclusive. The long-term impact of the hyperglycaemic intrauterine environment on offspring health is continuously being explored. Earlier studies seemed to suggest that there was no correlation between the rate of offspring obesity and maternal hyperglycaemia (Whitaker et al., 1998). However, a growing number of studies are now consistently implicating abnormal glucose tolerance during pregnancy and an increased prevalence of both obesity and metabolic dysregulation (including T2DM) in adolescent and adult offspring (Clausen et al., 2008; Zhen-Juan et al., 2017). These observations have thus led to the concept of intergenerational transmission of disease, with the diabetogenic environment in mothers with GDM perpetuating a cycle of metabolic abnormalities in mothers and offspring exposed to these environments. Consequently, these individuals further contribute to the rise in overall obesity and diabetes prevalence. Early screening and intervention are thus necessary to reduce maternal and fetal complications, providing an opportunity to modify the associated long-term health risks.

1.4.3 Management of GDM

Treatment of GDM largely focuses on intensive monitoring of maternal glucose levels so that normoglycemia is achieved and maintained throughout pregnancy. Optimal glycaemic targets are described as a fasting blood glucose level between 5.3 - 5.8 mmol/l, postprandial glucose level

less than 7.8 mmol/l at one hour and less than 6.7 mmol/l at two hours (NICE-Diabetes in pregnancy, 2015; Turok et al., 2003). The first line intervention strategy involves dietary or often referred to as medical nutritional therapy, along with lifestyle management. It is recommended that individualised dietary advice is given following assessment of each patient with the overall objective of attaining the appropriate dietary intake necessary for normal fetal growth and maternal health, whilst avoiding large carbohydrate loads at any one time. Dietary management may or may not be accompanied with light/moderate intensity physical activity (such as 20-30 minutes of aerobic training or walking 3 times per week) as this type of intervention has been shown to improve glucose levels and reduce the requirement for insulin (Colberg et al., 2016). Studies have shown that dietary treatment and regular monitoring of even mild gestational glucose intolerance, can ameliorate some of the negative consequences for new-borns, such as fewer large for gestational age (LGA) babies born in comparison to non-intervention group (Bonomo et al., 2005). Additionally, 80-90% of patients have been reported to achieve glycaemic control with dietary intervention alone (American Diabetes Association, 2015).

Patients who do not meet the glycaemic targets listed above, proceed to pharmacotherapies such as insulin or other oral anti-diabetic agents such as Metformin or the sulphonylurea, Glyburide (also known as Glibenclamide). An international consensus as to when pharmacological treatment should be initiated is yet to be ascertained but the National Institute for Health and Care Excellence (NICE) guidelines (2015) recommend the use of anti-hyperglycaemic drugs if glycaemic control is not achieved after 1-2 weeks of diet/lifestyle intervention (NICE-Diabetes in pregnancy, 2015).

Insulin is considered relatively low risk in pregnancy however, some women are hesitant about insulin administration and thus opt for alternative oral agents instead. Insulin analogues (i.e. insulin molecules with modified structure) are also becoming increasingly preferred over human insulin as they exhibit favourable pharmacokinetic properties. This includes rapid absorption and therefore allow for a faster peak insulin concentration to facilitate the lowering of large postprandial blood glucose increases common in GDM (Tamás and Kerényi, 2002). Additionally, these modified insulins are also associated with reduced risk of maternal hypoglycaemic episodes (Alfadhli, 2015) and therefore the subsequent risk of small for gestational age babies (SGA). Although the use of oral antidiabetic agents has not been approved by national regulating agencies, Metformin and Glyburide are commonly employed in the treatment of GDM. These drugs have differing mechanisms of action; Metformin predominantly improves insulin sensitivity and reduces hepatic glucose output whereas Glyburide stimulates insulin secretion. However, pregnancy outcomes and efficacy are generally similar for both drugs in most trials (Moore et al., 2010). The superiority of either drug is still controversial with one study revealing

approximately 35-46% and 16% of patients treated with Metformin or Glyburide respectively, ultimately requiring additional insulin for glycaemic control (Moore et al., 2010).

Assessment of maternal and fetal health outcomes remains a focus for research into the use of oral antidiabetic agents during pregnancy. Glyburide has been associated with increased incidence of macrosomia and metformin use is associated with a higher risk of prematurity (Coustan, 2007; Poolsup et al., 2014). Both drugs are readily transferred across the placenta with some reports of fetal drug concentrations similar or even higher to that of maternal levels (Hebert et al., 2009; R J et al., 2014; Vanky et al., 2005). Although no serious concerns regarding the short-term safety profile of their use has been highlighted, long-term safety data is still lacking.

1.5 Metabolic adaptations during pregnancy

In order to understand the pathophysiology of GDM it is first necessary to understand the changes in glucose homeostasis that occur during healthy pregnancy. During pregnancy, several maternal metabolic adaptations occur which correlate to the nutritional demands of both mother and fetus (Napso et al., 2018). The placenta serves as the interface between maternal and fetal circulations, orchestrating the sophisticated hormonal cues which are largely responsible for communicating the distinct phases of maternal metabolic adaptations. This includes the initial anabolic and later catabolic phases of pregnancy. The first two trimesters of pregnancy are generally considered the "anabolic phase" - involving enhanced maternal fuel/fat storage promoted by a combination of hormones (e.g. increased maternal prolactin and placental production of placental lactogen, progesterone and cortisol) stimulating lipid synthesis and storage whilst inhibiting lipid breakdown and inducing maternal hyperphagia. The "catabolic phase" represents the net effect of the breakdown of these fuel reserves and predominates the final trimester of pregnancy (Moyce and Dolinsky, 2018). An important mechanism responsible for this shift is the progressive increase in maternal insulin resistance that develops from mid to late gestation (Nahavandi et al., 2019).

The metabolic plasticity of pregnancy is commonly attributed to alterations in maternal insulin sensitivity. Early pregnancy has been associated with an increase in maternal insulin sensitivity index and first-phase insulin secretory response in a small cohort of pregnant women in comparison to pre-pregnancy (Powe et al., 2019). However, by late pregnancy total body insulin sensitivity is decreased by 45-70% in comparison to non-pregnant women (Freemark, 2006). The insulin resistance is also accompanied by increased fasting serum insulin and first phase insulin response as well as increased endogenous glucose production (Catalano et al., 1993; Sorenson and Brelje, 1997). Consequently, maternal insulin resistance results in prolonged post-prandial

elevated glucose in the maternal circulation, prioritising glucose availability for the developing fetus.

The augmentation to insulin secretion is independent of and prior to any changes in maternal insulin sensitivity, and it has thus been suggested that islet adaptations can occur as a direct consequence of circulating placental derived hormones (Powe et al., 2019). The importance of placental mediators establishing a diabetogenic environment throughout pregnancy through increasing maternal insulin resistance and regulating islet adaptations is made evident postpartum when a rapid increase in insulin sensitivity and reduction in insulin release is observed following delivery of the placenta (Mazaki-Tovi et al., 2011).

1.5.1 Placental regulation of the islets during pregnancy

As was earlier discussed in the introduction 1.1.2, multiple regulation levels are involved in modulating hormone secretion from pancreatic islets, ensuring the meticulous control of whole body glucose homeostasis. In addition to intra-islet hormone regulation, complex inter-islet - organ signalling has a significant role to play in influencing islet hormone secretion and function (Pia V. Röder et al., 2016). Many of these extra-pancreatic signals involve tissues which are glucose sensing and/or insulin sensitive tissues that interact with the pancreas via various hormones, neurotransmitters and cytokines. Well recognised islet axes exist between the brain, gut, liver and skeletal muscle with additional feedback mechanisms evident among adipose tissue (Pia V. Röder et al., 2016; Shirakawa et al., 2017). During pregnancy the maternal hormonal milieu changes throughout gestation to support the developing fetus. These changes are partly due to altered hormone release from maternal endocrine organs but are largely driven by the placenta which releases a wide range of hormones to regulate the maternal environment. The placenta communicates with a wide range of maternal tissues, including the pancreatic islets.

Although the placenta is a temporary endocrine organ during pregnancy, a unique placenta-islet axis is rapidly established during gestation to facilitate the physiological metabolic demand of maternal and fetal glucose homeostasis. The fetoplacental unit relies on glucose (the primary energy source) for sustained growth throughout pregnancy. Successful transfer of glucose across the placenta involves facilitated diffusion (via glucose transporter (GLUT) proteins) and relies on the establishment of a concentration gradient between maternal (high glucose) and fetal (low glucose) circulations. In order to modulate maternal glycaemia and optimise nutrient transfer, the placenta produces a number of biological signals which can act on pancreatic islets and insulin sensitive tissues to influence islet hormone secretion as well as maternal insulin sensitivity as discussed previously. Failure of the placenta to communicate the pregnant state and the endocrine response to support the metabolic requirements may result in pregnancy complications such as

gestational diabetes, fetal growth restriction or fetal overgrowth (Napso et al., 2018). The most studied interaction (s) between the placenta and pancreatic islet are those involved in the compensatory β-cell adaptations common to both rodent and human pregnancy. A more comprehensive review of the mechanisms involved in the placenta-islet cross talk during pregnancy will be discussed in section 1.5.5.

1.5.2 Maternal insulin resistance

The physiological factors and mechanisms responsible for the decrease in insulin sensitivity during pregnancy are not fully understood but are partially related to the metabolic effects of several placental-derived hormones/cytokines elevated in the maternal circulation during pregnancy (Seely and Solomon, 2003; Sonagra et al., 2014). Major drivers of the 2nd and 3rd trimester gestational insulin resistance include progesterone, placental lactogen, placental growth hormone and cortisol (corticosterone in rodents). Maternal plasma levels of these hormones rise as gestation progresses with peak levels reached during the second half of pregnancy, correlating to the onset of insulin resistance (Freemark, 2006). The potent effects of the pregnancy hormones mentioned above to induce severe peripheral insulin resistance have been demonstrated in studies both *in vitro* and *in vivo* and the cellular mechanisms are multifactorial.

Early studies by Ryan and Enns in the 1980's investigating the role of gestational hormones in the induction of insulin resistance demonstrated that glucose transport was decreased in primary cultures of female virgin adipocytes exposed to progesterone, cortisol and placental lactogen, but maximum insulin binding was mostly unaffected (Ryan and Enns, 1988). Further studies have also revealed an insulin resistant profile in adipose tissue and skeletal muscle of pregnant women compared to non- pregnant women. Decreased GLUT4 expression and translocation to the plasma membrane were attributable to the decreased glucose uptake in these tissues and represents a key mechanism in the blunting of post insulin receptor signalling and thus insulin resistance in pregnancy (Freemark, 2006; Okuno et al., 1995; Yamada et al., 1999). The decrease in insulin sensitivity in skeletal muscle is also mediated, in part, to a decrease in insulin-stimulated tyrosine kinase activity (by ~30-40%) and reductions in expression and tyrosine phosphorylation of insulin receptor substrate (IRS) proteins (Freemark, 2006). IRS proteins serve as immediate cellular substrates of activated insulin receptors and forms a complex with the insulin receptor. Formation of the insulin receptor-IRS complex acts as an adaptor protein or docking site for phosphoinositide-3 kinase (PI3K) (a lipid kinase), which is responsible for activating key downstream signalling effector proteins. Therefore, decreased activation/phosphorylation of IRS proteins will result in blunted insulin signalling. Placental growth hormone appears to induce insulin resistance through an alternative mechanism. Overexpression of placental growth hormone in transgenic mice to reach levels comparable to the 3rd trimester of pregnancy, resulted

in significant peripheral insulin resistance, attributable to the enhanced expression of the p85 subunit (the dominant negative competitor) of PI3K, thereby inhibiting the catalytic stability of PI3K (Barbour et al., 2002).

Other factors such as cytokines are also likely to be involved in promoting gestational insulin resistance. More recently, a significant role of the cytokine TNF-α has been implicated in the development of insulin resistance in pathological states such as obesity and diabetes along with pregnancy-induced insulin resistance (Borst, 2004). Studies looking at the longitudinal change in reproductive hormones have also demonstrated a significant correlation between circulating TNF-α and insulin resistance in late gestation suggesting TNF-α is a strong predictor of insulin resistance (Kirwan et al., 2002a). Additionally, an emerging role for adiponectin (a recently discovered adipokine known to have insulin-sensitising effects), has been linked to cellular mechanisms of insulin resistance, with decreased levels detected during pregnancy (Catalano et al., 2006).

1.5.3 Pancreatic islet adaptations to pregnancy

Whilst some degree of maternal insulin resistance is beneficial for fetal development the pancreatic islets must adapt to prevent the development of GDM. The metabolic environment of pregnancy thus requires a greater output of insulin to avoid persistent hyperglycaemia in the mother and excessive nutrient delivery to the fetus. The pancreatic islets fulfil these demands by undergoing compensatory adaptations which include regulated morphological and functional changes to β-cells.

It is now well recognised from several rodent studies and a limited number in humans that these adaptations are typically characterised by β-cell hyperplasia and hypertrophy (and possibly β-cell neogenesis) along with increased glucose-stimulated insulin secretion (as illustrated in Figure 1-4) (Moyce and Dolinsky, 2018; Rieck and Kaestner, 2010; Sorenson and Brelje, 1997). The expansion and hyperfunctionality of the β-cell is reversible, returning to pre-gestational levels postpartum. Disturbances to these adaptive mechanisms, leading to inadequate compensation of maternal insulin resistance, results in persistent hyperglycaemia and potentially overt gestational diabetes.

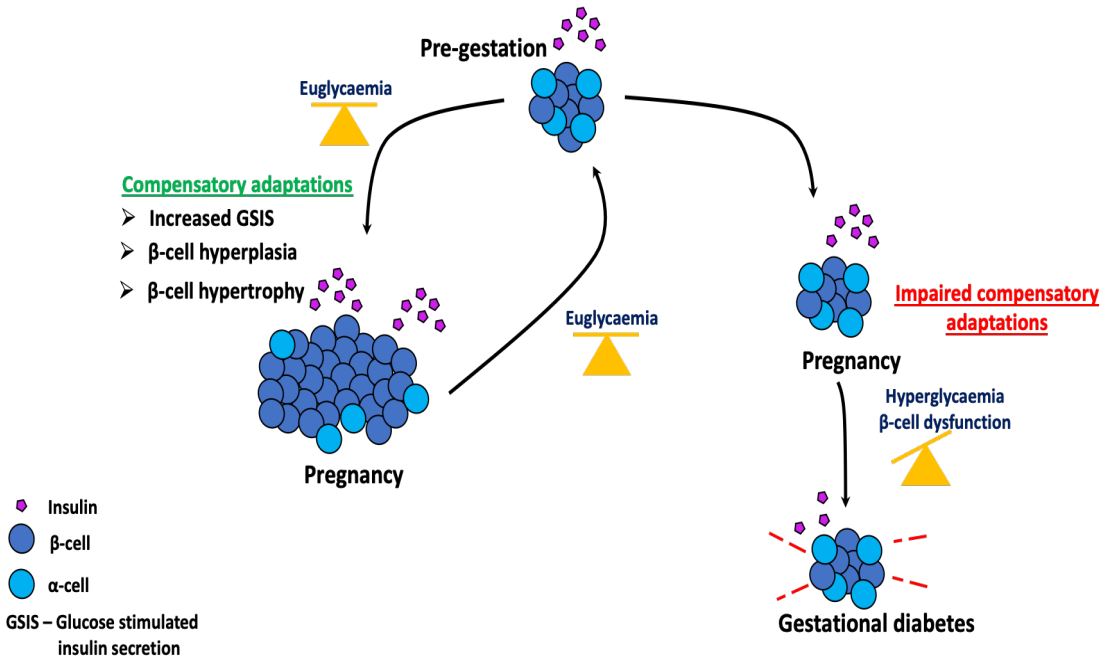

Figure 1-4 Schematic illustrating maternal β-cell compensatory adaptations during pregnancy. Pregnancy is associated with a progressive increase in maternal insulin resistance which is compensated by the β-cell by cellular process which include β-cell hyperplasia and hypertrophy accommodated with an increase in glucose-stimulated insulin secretion to ensure maternal euglycaemia. The morphological and functional changes to the β-cell return to pre-gestational levels postpartum. Impaired compensatory adaptations lead to persistent hyperglycaemia and β-cell dysfunction on the background of maternal insulin resistance and is thought to underpin a major pathophysiological mechanism of gestational diabetes.

The cellular processes regulating β-cell mass (i.e. the number and size of β-cells) include replication, death, hypertrophy and neogenesis (Ernst et al., 2011). In both rodents and humans, β-cell mass is relatively static during adulthood particularly in human β-cells which are more resistant to replication than rodent β-cells, owing to extremely low rates of proliferation and neogenesis (Wang et al., 2015). However, in rodent pregnancy the rate of β-cell proliferation and apoptosis is altered to control the adaptive expansion of β-cell mass (Rieck and Kaestner, 2010). The dynamics of β-cell mass expansion in mice reveals a peak in β-cell mass at around day 16 of gestation which is accompanied by slightly earlier increases in β-cell proliferation and hypertrophy during the first two-thirds of pregnancy. β-cell apoptosis increases near the end of term (from day 18 into the early postpartum period) to contract β-cell mass to pre-gestational levels (see Figure 1-5). Experimental studies in rodents have shown that by the end of gestation

rodents display a 2- 4 fold increase in β-cell mass (Ernst et al., 2011; Parsons et al., 1992; Rieck and Kaestner, 2010; Sorenson and Brelje, 1997).

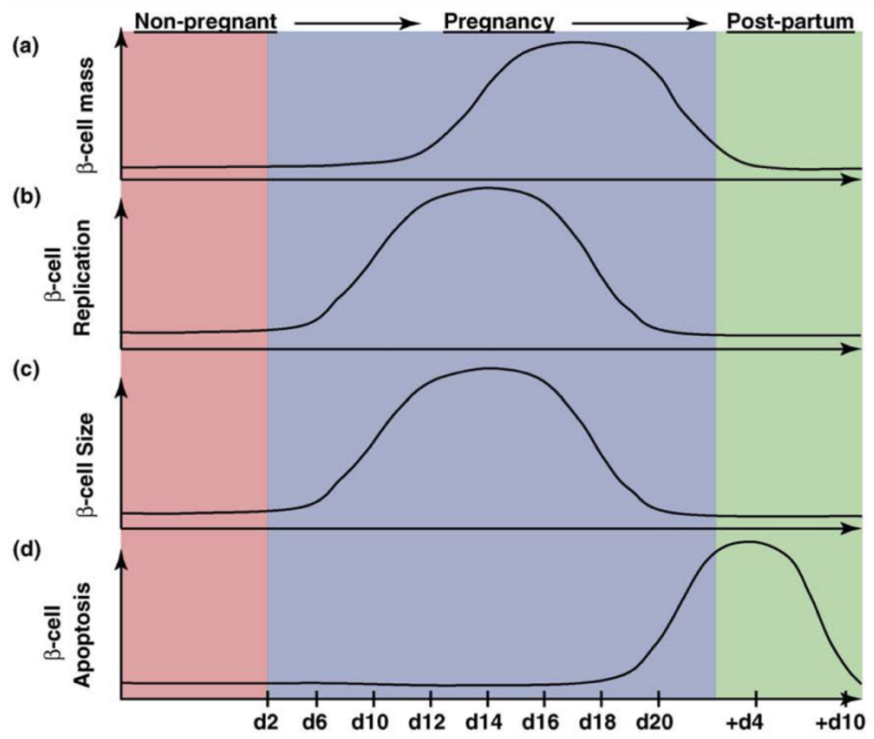

Figure 1-5 Dynamics of β-cell mass expansion during mouse pregnancy. Approximate changes in the processes leading to β-cell mass expansion during pregnancy (A) are largely driven by an increase in β-cell hyperplasia (B) and hypertrophy (C). Nearing the end of term (d19), increase in β-cell apoptosis facilitates the return of β-cell mass to non-pregnant levels (D). Image taken from (Rieck and Kaestner, 2010).

Data on β-cell adaptations in human pregnancy is scarce due to the limited availability of human autopsy samples and confounding factor of sample heterogeneity. Recently, Butler and colleagues highlighted that the increase in maternal β-cells in human gestation was much smaller (1.4 – 2 fold) than that typically observed in rodents, based on the evaluation of autopsy samples from women who died whilst pregnant (Butler et al., 2010). Furthermore, no difference in β-cell Ki67 (a marker of cell proliferation) was detected between age-matched pregnant and non-pregnant women. However, it was observed that in pregnancy there were more small islets and an increase in scattered β-cells and duct cells positive for insulin, suggestive of neogenesis of β-cells from

other cell types as opposed to the characteristic β-cell proliferation in rodent pregnancy (Butler et al., 2010). It thus emerges that there may be different mechanisms responsible for the expansion of β-cells between humans and rodents during pregnancy (Baeyens et al., 2016). The inability to study islet anatomy *in vivo* in humans or assess neogenesis directly on autopsy samples makes it difficult to conclusively determine the mechanisms involved. More is yet to be investigated to fully characterise the β-cell adaptations in human pregnancy.

In addition to increased β-cell mass, one of the most notable functional adaptations of the hyperdynamic pool of β-cells during pregnancy is an enhanced glucose stimulated insulin secretory response (peaking midway through gestation) (Pasek and Gannon, 2013). Increases in glucokinase activity, glucose metabolism and insulin synthesis enable the elevation in insulin secretion (Green and Taylor, 1972; Parsons et al., 1992; Sorenson and Brelje, 1997; Weinhaus et al., 2007). An important component of the enhanced insulin secretion is the increased sensitivity of the β-cell to glucose as a result of increased expression and activity of the glucose-sensing enzyme glucokinase. In perfused pancreata of pregnant rats the glucose-stimulation threshold was significantly decreased from 5.7 mM glucose to 3.3 mM glucose by day 12 of gestation. Concomitant above threshold insulin secretion was significantly increased by day 12, peaking at day 15 of gestation. Intriguingly, glucose threshold and insulin secretion returned toward normal physiological levels by day 20 (Parsons et al., 1992). Hyperinsulinemia is exhibited both in human and rodent pregnancy with a progressive increase (~ 75%) in fasting serum insulin reported in rodent models and thus provides further evidence of hyperfunctionality of the β-cells during the pregnant state (Genevay et al., 2010; Sorenson and Brelje, 2009; Spellacy et al., 1965).

1.5.4 Pathophysiology of GDM

Although the normal insulin resistance of pregnancy is anticipated and countered by β-cell adaptations, pregnancies complicated by GDM are thought to have impaired β-cell adaptative mechanisms on a background of exaggerated insulin resistance. Knowledge of the specific molecular pathways disrupted by GDM is still lacking but β-cell dysfunction and tissue insulin resistance represent critical components underlying the pathophysiology of GDM (Plows et al., 2018). The major consequence of maladaptive responses of the β-cell to pregnancy is insufficient insulin production to meet the increased maternal requirements. Studies in women with GDM reveal that β-cell function is reduced by 30 - 70% indicating that β-cells are unable to compensate the pregnancy associated insulin resistance (Homko et al., 2001; Lain and Catalano, 2007; Nguyen-Ngo et al., 2019; Xiang et al., 1999). In addition to relatively lower insulin responses to glucose, patients with GDM also exhibit additional defects in insulin signalling (Lain and Catalano, 2007). In fact, the decrease in insulin sensitivity in women with GDM compared to

pregnant normoglycemic women is even more sever. In skeletal muscle specifically, the rate of insulin stimulated glucose uptake is reduced by 54% in GDM pregnancies compared to a reduction of only 32% in pregnant women with normal glucose tolerance (NGT) (Catalano, 2014). The further decreased insulin sensitivity in women with GDM has been associated with decreased tyrosine phosphorylation of the β-subunit of the insulin receptor (IR) despite no significant differences in IR binding or abundance (Barbour et al., 2007; Friedman et al., 1999). Additionally, women with GDM display lower protein expression and tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1) in adipose tissue and skeletal muscle (Catalano et al., 2002; Nguyen-Ngo et al., 2019; Shao et al., 2002). Tyrosine phosphorylation of the IR-β-subunit and IRS-1 is required for the activation of downstream effector proteins involved in the translocation of GLUT4 to the cell surface for glucose uptake. Subsequently, these alterations to insulin signalling in patients leads to a decrease in insulin stimulated glucose uptake beyond that of normal pregnancy and results in clinical hyperglycaemia. Furthermore, women with GDM display a greater proportion of serine phosphorylated IRS-1 (reducing its ability to act as an insulin receptor substrate), further inhibiting insulin signalling and contributes to the insulin resistance in GDM.

The stimulus for the above described changes in insulin sensitivity and β-cell function are not fully understood, however, given the important influence of placental related hormones in the physiological alterations to maternal metabolism, it is suggested that placental dysfunction may also play a role in the pathophysiology of GDM. For example, compromised placental function by chronic low-grade inflammation could possibly amplify signals that exacerbate the insulin resistance in GDM and/or impair signals which support islet adaptive mechanisms in normal pregnancy. Emerging evidence has therefore suggested that the development of GDM is associated with dysregulated expression of proinflammatory cytokines which may impair insulin sensitivity and secretion. Increased circulating levels of proinflammatory cytokines interleukin - 6 (IL-6) and TNF- α have thus been reported in pregnancies complicated by GDM when compared with normal pregnancies (Atègbo et al., 2006; Winkler et al., 2002; Zhang et al., 2018). The placenta represents the main source of the elevated levels observed in the maternal circulation in GDM, as perfusion studies in human placenta revealed 94% of TNF-α produced is secreted into the maternal circulation (Kirwan et al., 2002a). TNF-α has commonly been linked to mechanisms of insulin resistance characterised in obesity, T2DM and normal pregnancy (Borst, 2004; Swaroop et al., 2012). Therefore, it is possible that TNF-α along with other cytokines may be involved in propagating insulin resistance leading to GDM.

It is evident that GDM develops as a result of complex and variable interactions between placental factors, inflammation, diet, genetics and environmental influences which may provoke insulin resistance, β-cell dysfunction or both as displayed in Figure 1-6. Despite the traditional view that a combination of insulin resistance and β-cell impairment contribute to the pathogenesis of GDM,

emerging evidence suggest that differences in the underlying pathophysiology between GDM patients exists. Powe and colleagues have thus demonstrated that in a cohort of pregnant women (24-30 weeks' gestation) 51% of women diagnosed with GDM primarily had deficiency in insulin sensitivity, 30% primarily had a deficiency in insulin secretion and 18% had a mixed pathophysiology of both insulin sensitivity and secretion (Powe et al., 2016). This highlights the possibility that subgroups of GDM patients exist and the need to understand much more about the heterogenous pathophysiological processes underlying hyperglycaemia in GDM. However, the main obstacles in progressing our understanding of the pathophysiology of GDM include a lack of animal models that fully recapitulate the human disease as well as the scarcity of tissue and organ samples, particularly pancreatic tissue, from pregnant women with and without GDM. Nevertheless, experimental studies in rodents investigating the potential signals/regulatory pathways involved in GDM/ islet adaptations are facilitating a clearer picture in general of the disease.

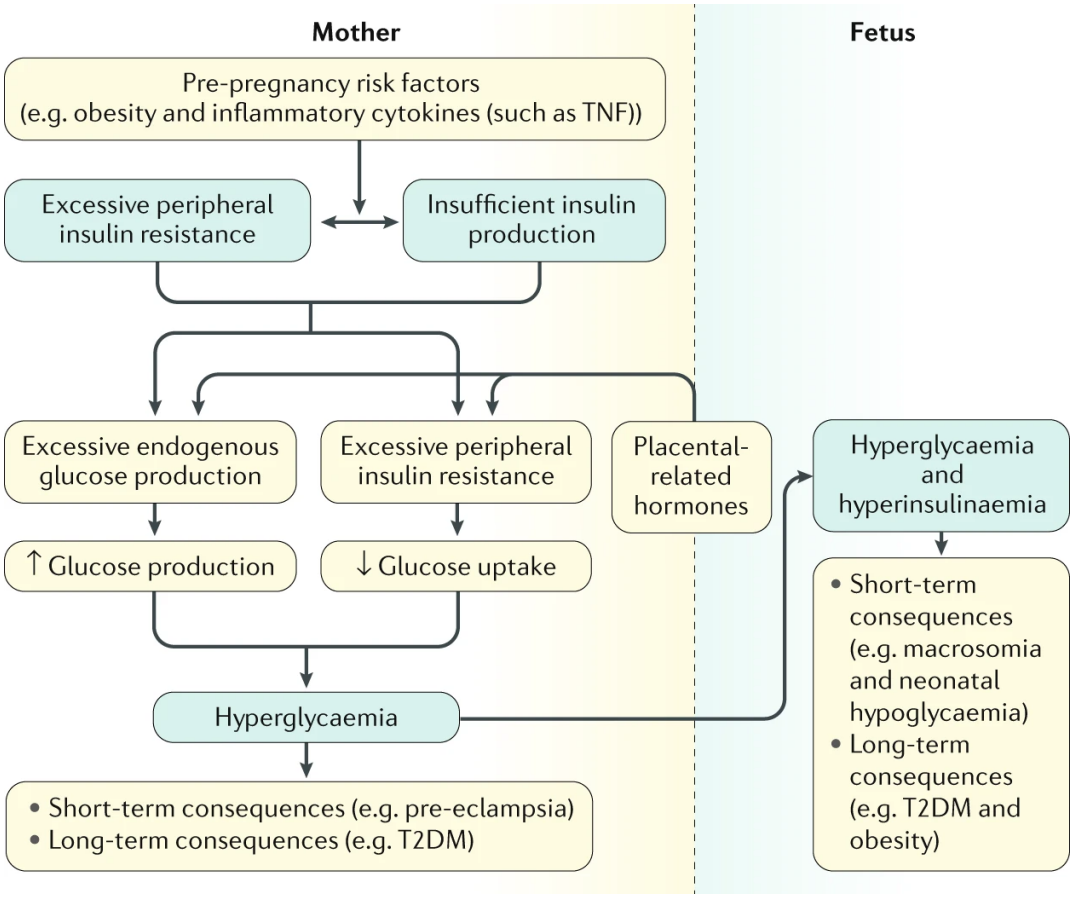

Figure 1-6 Pathophysiology of GDM. Excessive peripheral insulin resistance and β-cell dysfunction are critical pathophysiological components of gestational diabetes with obesity, personal history of GDM and genetic predispositions representing pre-pregnancy risk factors. The pregnancy environment including maternal peripheral insulin resistance and contribution of placental-related hormones exacerbate metabolic disturbances such as excessive peripheral insulin resistance and glucose production as well as insufficient insulin production. Subsequent reduction in glucose uptake and concomitant increase in glucose production result in clinical hyperglycaemia which can have both short-term and long-term health consequences for both mother and fetus. Image taken from (McIntyre et al., 2019)

1.5.5 Signals mediating pancreatic islet adaptions to pregnancy

The progressive decline in maternal insulin sensitivity during pregnancy has long been considered the principal inducer of pregnant β-cell adaptations. Growing evidence now supports the current view that circulating factors in maternal serum stimulate the expansion of β-cell mass and function. Evidence of adaptations preceding the development of insulin resistance and a rise in pituitary and placental hormones parallel to β-cell compensation further support this model (Baeyens et al., 2016; Parsons et al., 1992). The transcriptional response of the islet to pregnancy

in mice has highlighted the complexity in the many potential pathways involved in the β-cell adaptations with nearly 2,000 genes identified to be differentially expressed throughout gestation (Rieck et al., 2009). Of the genes identified, the prolactin receptor (PRLR) (which functions as a receptor for both prolactin (PRL) and placental lactogen (PL)) displayed almost 3 -fold increased expression compared to non-pregnant control islets. Furthermore, the increased expression was evident as early as day 10.5 (when β-cell replication is thought to initiate) and at day 14.5 (concurrent with the peak of β-cell proliferation) suggesting lactogenic signalling plays an important role in mediating the adaptive response (Rieck et al., 2009). Thus, researchers investigating the β-cell adaptations during pregnancy have focused their attention mostly on the role of circulating lactogenic hormones; placental lactogen and prolactin, and the β-cell response to these signals.

Multiple *in vitro* and *in vivo* studies examining the effects of PL and PRL on islets in rodent models have demonstrated that lactogens increase insulin secretion, β-cell proliferation, survival and mass (Brelje et al., 1993; Ernst et al., 2011; Sorenson et al., 1993). Studies by Vasavada and colleagues have demonstrated the potent insulinotropic and mitogenic effects of PL. Transgenic mice overexpressing PL specifically in β-cells display an increased rate of proliferation as well as β-cell size and thus display a relatively hypoglycaemic phenotype in fasting and non-fasting conditions (Vasavada et al., 2000). Conversely, global deletion of the PRLR in mice results in decreased overall β-cell mass coupled with lower insulin mRNA and content as well as a blunted glucose-stimulated insulin secretory response when compared with their wild-type littermates (Freemark et al., 2002). More recently, Huang (2009) and a separate study by Banerjee and colleagues (2016), has been able to directly show the contribution of lactogenic signalling in modulating pregnancy-induced β-cell adaptations using heterozygous or conditional β-cell PRLR knock-out mice respectively. Taken together, these studies highlighted that that loss of PRLR signalling specifically in β-cells results in glucose intolerance or gestational diabetes mellitus as a consequence of failure to expand β-cell mass or increase functional demand (Banerjee et al., 2016a; Huang et al., 2009).

A growing body of data is now beginning to piece together the downstream signalling mechanisms of PRLR activation and regulation of these adaptations. As mentioned previously, hundreds of genes display altered expression patterns in the pregnant islet (especially around gestational day 13-15 – the peak of adaptations) (Ernst et al., 2011; Layden et al., 2010). Among these genes, tryptophan hydroxylase 1 and 2 (TPH1/2), are commonly found to be significantly upregulated in pregnant islets (Layden et al., 2010; Rieck et al., 2009). TPH1 and 2 are two isoforms of the enzyme that control the rate-limiting step of serotonin synthesis. Elegant studies have demonstrated a strong lactogen-dependent upregulation of serotonin biosynthesis and

secretion with further evidence in mouse models suggesting that increased islet serotonin during pregnancy drives β-cell expansion (Kim et al., 2010; Schraenen et al., 2010). Complementary to this, islet serotonin receptor expression is altered in the pregnant islet, with upregulation of the $G_{\alpha q}$-linked serotonin receptor, 5-hydroxytryptamine receptor-2b (Htr2b) during pregnancy which normalises just before parturition (Kim et al., 2010). Blocking Htr2b signalling in pregnant mice also blocked β-cell expansion and caused glucose intolerance providing evidence of the integrated signalling pathways linking lactogen signalling to β-cell adaptations in pregnancy. The mouse genome consists of 14 different serotonin receptor genes and several subsequent studies using rodent models deficient in alternative serotonin receptor isoforms have implicated a pregnancy-specific role for Htr3 signalling in compensatory β-cell mechanisms as well (Ohara-Imaizumi et al., 2013).

Currently, there is still debate about which functional serotonin receptor could play a role during pregnancy in mouse islets. Findings from transgenic mouse models and the impact to pregnancy physiology needs to be confirmed. Complete loss of islet serotonin signalling does not halt β-cell proliferation in pregnant mice, indicating that complementary pathways may exist for β-cell adaptations. Furthermore, despite the vast amount of data supporting a significant role for lactogen-induced β-cell compensation, comparable levels of PL and PRL have been reported between non-obese pregnant women with GDM (representing a potential state of maladaptive β-cell responses to pregnancy) and age-matched healthy pregnant women (Grigorakis et al., 2000). Despite the focus within the literature on the lactogenic hormones, other hormones are likely to play a role in regulating the islet adaptations to provide a well-balanced reprogramming of β-cell physiology in pregnancy (Moyce and Dolinsky, 2018).

Recently, a role for placental kisspeptin in the physiological islet adaptations to pregnancy has been demonstrated (Bowe et al., 2019). Kisspeptins are a family of hypothalamic neuropeptides well known for regulating puberty and reproductive function signalling via its cognate GPCR, GPR54/KISS1R (Clarke et al., 2015; de Roux et al., 2003; Kotani et al., 2001; Seminara et al., 2003). Under most physiological circumstances circulating kisspeptin levels are extremely low (Horikoshi et al., 2003a). Improper kisspeptin function or low hypothalamic kisspeptin levels can attenuate reproductive development as well as cause infertility (Lapatto et al., 2007; Mumtaz et al., 2017; Seminara et al., 2003). Having high levels of kisspeptin is not yet related to any conditions, however studies have shown that high circulating levels of the hormone is found in the maternal circulation during pregnancy, increasing several thousand-fold in humans (Dhillo et al., 2006; Horikoshi et al., 2003a). Additionally, GPR54 is highly expressed in pancreatic islets and *in vitro* studies have also revealed the stimulatory effects of exogenous kisspeptin on insulin release (Bowe et al., 2012, 2009; Hauge-Evans et al., 2006; Kotani et al., 2001; Schwetz et al.,

2014) supporting a role for placental kisspeptin in potentially modulating the islet adaptation to pregnancy. This hypothesis was thus investigated by Bowe and colleagues utilising *in vivo* mouse models and clinical samples from pregnant women as described below.

Pharmacological blockade of endogenous kisspeptin in pregnant mice resulted in impaired glucose homeostasis which was associated with a reduced insulin response to glucose. Subsequent generation of a β-cell -specific GPR54 knockdown mouse model confirmed these effects on glucose tolerance were directly mediated through β-cell GPR54 as glucose intolerance was exhibited in these mice during pregnancy with no phenotype observed outside of pregnancy. Deletion of β-cell GPR54 on pregnant β-cell mass also demonstrated a significant reduction in pregnancy induced β-cell proliferation as measured by positive bromodeoxyuridine (BrdU) staining. Furthermore, women with GDM displayed significantly lower levels of circulating kisspeptin compared to pregnant women without GDM (Bowe et al., 2019). All these data are consistent with a role for kisspeptin dependent β-cell adaptive responses to pregnancy and demonstrates the importance of signals complementary to lactogenic signalling in driving islet adaptations.

Limited studies have begun to address the role of other hormones/growth factors that may be involved in the pregnancy induced β-cell adaptations. Candidate signalling mechanisms include hepatocyte growth factor (HGF) and its cognate receptor, c-Met. HGF, secreted by fibroblasts, is among a group of factors which display potent angiogenic/mitogenic ability and is an insulinotropic agent for the β-cell (Demirci et al., 2012; Garcia-Ocaña et al., 2000). Circulating HGF is also markedly increased during pregnancy in humans due to increased production and secretion by the placenta (Horibe et al., 1995). As with the model of local islet serotonin signalling stimulating β-cell adaptations, circulating or locally secreted HGF is suggested to also participate in driving β-cell mass expansion (Demirci et al., 2012). However, deciphering the role of HGF/c-Met signalling in the β-cell during pregnancy is still required.

More generally, the dynamic interaction between the placenta and islets has been recently studied in mice by Drynda and colleagues through the identification of an atlas of placental- derived ligands ("placenta secretome") and complementary islet GPCRs ("islet GPCRome"), revealing numerous potential routes of interaction between the placenta and β-cells (Drynda et al., 2018). Upon comparing similar patterns of expression with those of the lactogenic hormones (i.e. increased expression of islet GPCR and the placental ligand (s) during the active period of β-cell adaptive responses), a number of functional GPCR/ligand combinations were identified including corticotropin releasing hormone (CRH) and its cognate receptors. Both placental *Crh* and islet corticotropin releasing hormone receptor 1 (*Crhr1)* were upregulated on gestational day 12

compared to gestational day 18 (Drynda et al., 2018). Published studies have previously reported the ability of CRH to influence islet function though the physiological role for these effects were unclear (Huising et al., 2010; O'Carroll et al., 2008). Given these observations, the CRH system seems an interesting candidate to investigate as a potential signal that may contribute to maintaining β-cell function during gestation.

1.6 Corticotropin releasing hormone (CRH) and Urocortins (UCNs)

1.6.1 The CRH peptide family

Corticotropin releasing hormone (CRH) (also known as corticotropin releasing factor - CRF) is a hypophysiotropic neuroendocrine hormone which was first isolated and characterised in 1981 (Vale et al., 1981). The 41-amino acid peptide hormone is derived from the 196-amino acid precursor, preproCRH, following cleavage of its C-terminal region. Human and rat CRH (h/rCRH) share identical amino acid sequences whereas ovine CRH, the species where CRH was originally isolated and characterised from, varies by 7 amino acids (Seasholtz et al., 1991; Vale et al., 1981). More recently, the discovery of three novel CRH-like peptide forms has expanded members of the CRH family to now include CRH, Urocortin 1 (UCN1), Urocortin 2 (UCN2) (or stresscopin-related peptide) and Urocortin 3 (UCN3) (or stresscopin) (Lewis et al., 2001a; Reyes et al., 2001; Joan Vaughan et al., 1995). These peptides were identified due to their sequence homology to CRH with UCN1, a 40-amino acid peptide displaying ~45% homology and UCN2 and -3 (both 38- amino acid peptides) sharing ~34% and ~30% identity to h/rCRH respectively (Hauger et al., 2003a). The distinct genes for CRH and urocortins are highly conserved across species indicating the physiological importance of this neuroendocrine signalling system as a survival mechanism.

Initial observations highlighting the hypothalamic extract - CRH, as a potent inducer of the release of adrenocorticotropin hormone (ACTH) from the anterior pituitary after exposure to various stressors, was fundamental in elucidating the role of CRH as the primary regulator of the stress response (Guillemin and Rosenberg, 1955; Saffran et al., 1955; Taché and Brunnhuber, 2008). The CRH family of peptides are thus now recognised as playing biologically diverse roles in coordinating responses to stress. The widespread anatomical distributions of CRH and urocortins within the central nervous system (CNS) correlates well with this system being able to facilitate a range of physiological functions. CRH is abundantly distributed throughout the CNS however, it is predominantly expressed in the paraventricular nucleus (PVN) of the hypothalamus. Expression has also been detected in the cerebral cortex, amygdala and hippocampus. UCN1 expression is mostly limited to cell bodies of the Edinger-Westphal nucleus (EWN) and although UCN2 and -3 display more discrete patterns of distribution within brain regions, expression of

the peptides have been reported in the arcuate nucleus of the hypothalamus and amygdala, respectively (Dautzenberg and Hauger, 2002; Ryabinin et al., 2012). Expression of these neuropeptides was once thought to be restricted to the brain and pituitary however, studies have revealed differential expression patterns for CRH and urocortins in peripheral tissues (Boorse and Denver, 2006). CRH immunoreactivity has been detected in the adrenal gland, heart, intestine, pancreas and liver (Boorse and Denver, 2006; Petraglia et al., 2010). Similarly, the urocortins have been detected in peripheral tissues, including the heart and adipose tissue for UCN1, adrenal gland, placenta, heart and a high expression in skeletal muscle and skin for UCN2 and the gastrointestinal tract and pancreas for UCN3 (Chen et al., 2004; Petraglia et al., 2010).

1.6.2 CRH receptors

To date, two membrane bound, G-protein coupled receptors (GPCRs); CRHR1 and CRHR2 have been isolated. Additionally, a soluble, structurally unrelated 37kDa glycoprotein; CRH-binding protein (CRH-BP), has also been reported (Chen et al., 1993; Lovenberg et al., 1995b; Orth and Mount, 1987; Seasholtz et al., 2002). CRHR1 and 2, approximately 415-420 and 397-438 amino acid proteins, respectively, are encoded by two separate genes, exhibiting approximately 70% amino acid sequence homology. They belong to the class B subfamily of GPCRs which include receptors for growth hormone releasing hormone, vasoactive intestinal peptide, parathyroid hormone as well as glucagon (Dautzenberg and Hauger, 2002). The CRH receptors display distinct binding characteristics and pharmacological profiles owing to the modest degree of homology (~40%) in their N-terminal extracellular domain (Hauger et al., 2003b). This divergence results in differences in ligand selectivity with CRH and UCN1 binding to both receptors (although UCN1 shows a greater affinity than CRH to CRHR2: 0.41nM vs 17nM for UCN1 and CRH respectively) whereas UCN2 and UCN3 are selective for CRHR2 only (Grammatopoulos, 2012; Seasholtz et al., 2002).

In accordance with all GPCRs, activation of CRH receptors results in coupling to heterotrimeric GDP/GTP bound proteins with both receptor subtypes primarily coupling to $G_{\alpha s}$ (Grammatopoulos, 2012). Subsequent generation of the intracellular second messenger, cyclic adenosine monophosphate (cAMP) following activation of adenyl cyclase, results in a diverse array of downstream signalling events including the post-translational modification of target proteins by protein kinase A (PKA) and regulation of gene transcription by cAMP response element-binding (CREB) proteins. The receptors however, do appear to display signalling promiscuity as they have been reported to couple to multiple G-proteins (Grammatopoulos et al., 2001) which not only exhibit receptor specificity but are also agonist- and tissue- specific. For example, activation by CRH of stably transfected HEK293 cells with CRHR1 can stimulate cAMP generation as a result of $G_{\alpha s}$ activation. However, CRH failed to stimulate the ERK1/2

pathway that was induced by UCN1 in the same cell system (Bonfiglio et al., 2011). The diverse and unique biological effects of CRHR signalling has also been demonstrated in human myometrium where activation of different CRHR signalling cascades have been demonstrated before and after the onset of labour (You et al., 2012). The mechanisms determining the versatility in signal transduction are still unknown but it is evident that receptor type, agonist specificity and cellular context play a fundamental role.

CRHR1 and CRHR2 also display differential expression patterns throughout the CNS and periphery which are generally non-overlapping again further supporting the idea that there may be a wide range of physiological outcomes/consequences in response to receptor activation. The main locus for CRHR1 expression is in the brain with high densities found in the pituitary gland (Reul and Holsboer, 2002). Conversely, CRHR2 reveals a more discrete expression profile in brain regions in comparison to a relatively dominant peripheral expression pattern (Hiroi et al., 2001; Lovenberg et al., 1995a). CRHR1 mRNA has been detected in gonadal tissue, adrenal glands, placenta as well as within pancreatic islets. The distribution of CRHR2 extends from gastrointestinal tissue, cardiac myocytes with expression of the receptor also observed in skeletal muscle and skin (Dautzenberg and Hauger, 2002; Hillhouse and Grammatopoulos, 2001).

The complexity of the CRH system is exemplified further by the multiple receptor isoforms which exist as a result of extensive sequence splicing. The primary functional CRHR1 isoform is CRHR1α, with at least an additional 7, non-functional splice variants reported so far for this receptor subtype (Slominski et al., 2004). Three functional splice variants of the CRHR2 have been reported with rodents possessing the CRHR2α and 2β isoforms only. However, a third isoform, CRHR2γ, thought to be exclusive to humans, is yet to be detected in any other species (Kostich et al., 1998; Lovenberg et al., 1995a) (see Figure 1-7). As expected, the receptor isoforms also have distinct tissue distributions and moreover, there appears to be a preferential anatomical distribution between species as the main splice variant found in the periphery for rodents is CRHR2β. In humans, CRHR2α appears to be the predominant peripheral isoform (Grammatopoulos and Chrousos, 2002).

Unlike the membrane bound receptors, CRH-BP, originally isolated from human plasma (Orth and Mount, 1987) is a secreted glycoprotein structurally unrelated to CRHRs and is thought to modulate the action and bioavailability of CRH and CRH-related peptides. Humans as well as most other species including rat, show well characterised expression of CRH-BP in brain regions with CRH-BP expression overlapping with CRH and CRHRs. This co-expression suggests that the CRH-BP has a modulatory effect on CRH receptor interaction (Kemp et al., 1998). Since its initial isolation, CRH-BP has also been detected in human placenta and liver, a phenomena believed to be unique to primates (Behan et al., 1995). CRH-BP in human placenta is postulated

to prevent inappropriate stimulation of the stress axis by placental CRH but its detection in male and non-pregnant females (with the primary source being the liver in this case) suggest that the binding protein serves other physiological functions outside of pregnancy (Behan et al., 1995; Kemp et al., 1998). Although CRH-BP was originally not believed to be expressed in non-neuronal tissue in rodents, CRH-BP transcripts and protein have been detected in rat adrenals (Chatzaki et al., 2002) though its peripheral role is still unclear. One suggested role of the binding protein is in an inhibitory capacity (i.e. sequestering CRH ligands away from the receptor and thus decreasing receptor activation). It has also been proposed that CRH-BP could alternatively have enhancing activity for the CRH system. For example, the binding protein could function to protect/preserve CRH ligands from degradation, "delivering" CRH peptides to receptors in target tissues or even mediate effects directly itself (Seasholtz et al., 2002). With CRH-BP displaying a higher affinity for CRH and UCN1 in comparison to UCN2 and UCN3, it is still unclear as to what specific function this glycoprotein plays within this system. Even greater complexity of the CRH family is made apparent by the recently identified rodent-specific soluble α-isoform of CRHR2 (sCRH-R2α) (Chen et al., 2005). Similar to the CRH-BP, sCRH-R2α binds CRH and UCN1, with little/no affinity for UCN2 (Vandael and Gounko, 2019). Again, limited information on the role of this soluble CRHR2 is known.

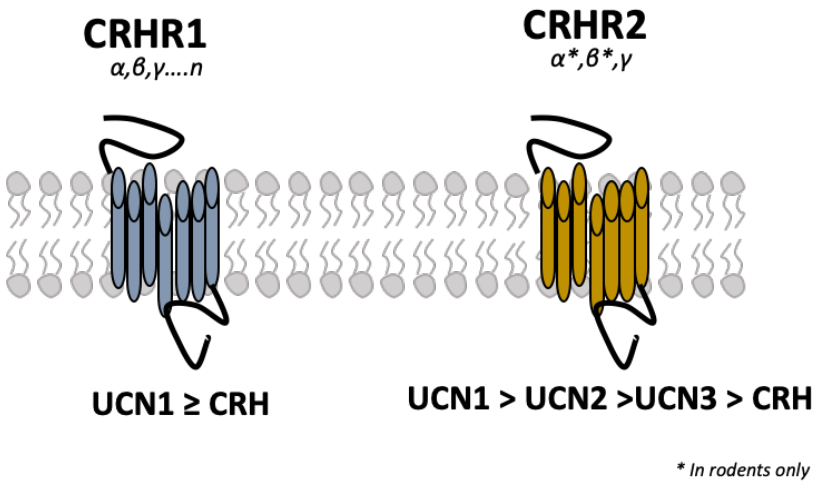

Figure 1-7 Summary of CRH receptors and relative order of affinity of CRH-family peptides. Two major types of CRH receptor exist; CRHR1 and CRHR2, both of which are GPCRs. Several isoforms of each receptor exist as a result of sequence splicing. CRHR1α is the only functional isoform for CRHR1. CRHR2α and 2β are the predominant isoforms in both humans and rodents. CRH has specificity for the CRHR1 and has the least affinity towards CRHR2. UCN1 can activate both CRHR1 and CRHR2, and UCN2 and -3 show specificity for CRHR2 and do not appear to have affinity for CRHR1 at physiologic concentrations.

1.6.3 The hypothalamic- pituitary-adrenal (HPA) axis

The hypothalamic CRH system provides the ability for organisms to adapt to various actual or even perceived homeostatic threats, commonly referred to as "the stress response". Activation of the HPA axis integrates hypothalamic and pituitary neuroendocrine signalling to peripheral signalling in the adrenal glands. The fundamental outcome being the mediation of an appropriate, adaptive whole-body physiological response to stress, such as energy mobilisation and distribution in multiple organ systems (Herman et al., 2016). CRH is the principal regulator of the HPA axis. Physical or psychological stressors evoke the rapid secretion of CRH from the parvocellular paraventricular nuclei (PVN) of the hypothalamus into the blood vessels connecting the hypothalamus and the pituitary (hypophysial portal circulation). Upon binding to its cognate receptors in the anterior pituitary, particularly CRHR1 located on corticotroph cells, biosynthesis of pro-opiomelanocortin (POMC) results in the production and release of ACTH into the systemic circulation. ACTH acts on the cortex of the adrenal gland to stimulate the synthesis and release of glucocorticoids. The released glucocorticoids are mainly cortisol in humans or corticosterone in rodents which have a wide range of biological effects to support the stress-induced adaptations. Concurrently, the released glucocorticoids serve as negative regulators of the HPA axis, supressing the secretion of both ACTH and CRH at the pituitary and hypothalamic level respectively and thus restores the system back to baseline levels (see Figure 1-8).

Dysregulation of the HPA axis and thus cortisol levels, can lead to adverse health consequences such as Cushing's syndrome in the case of too much cortisol or Addison's disease with too little cortisol secretion (Raff et al., 2014). Alterations to the CRH system such as chronic secretion of CRH are implicated in stress-related affective disorders such as anxiety and depression (Risbrough and Stein, 2006). The relative contribution of the urocortin peptides in the HPA axis extends to a modulatory role, fine-tuning stress responses, particularly stress-related behavioural responses with complementary and sometimes contrasting effects to CRH. The little neuroanatomical overlap between CRH and urocortins suggests that although part of the same system, the urocortins may have distinct roles within the HPA axis and possibly have an important role in moderating stress-recovery mechanisms (Neufeld-Cohen et al., 2010).

Although the primary function of CRH and urocortin peptides is in regulating the HPA axis, extended functions of CRH/CRHRs beyond the HPA-axis exist (Herman et al., 2016). In fact, neuropeptides classically associated with the HPA axis are increasingly being reported to have novel roles in other tissue systems including in wound healing and in skin physiology (Rassouli et al., 2018, 2011).

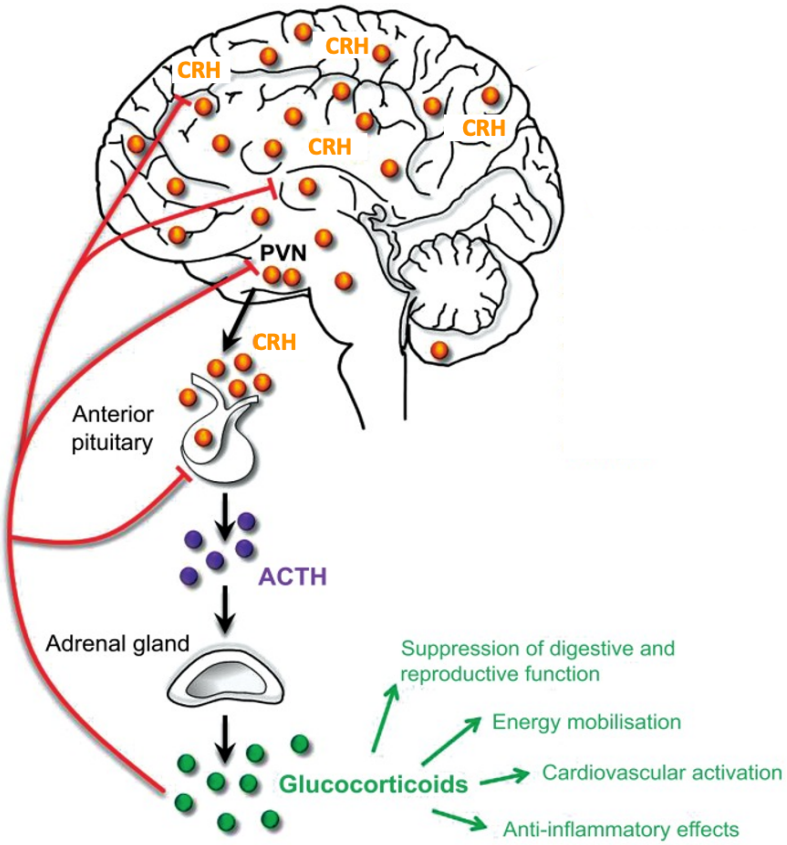

Figure 1-8 Regulation of the HPA- axis. Physiological or psychological stress is followed by a series of events led by the release of CRH from the paraventricular nucleus (PVN) of the hypothalamus into the hypophysial portal vasculature. The neuropeptide then binds to and activates CRHRs (on corticotroph cells in the anterior pituitary) stimulating the release of adrenocorticotropin hormone (ACTH). ACTH is subsequently released into the systemic circulation and in turn triggers the synthesis and release of glucocorticoids from the adrenal cortex. These hormones mediate various physiological and metabolic responses, preparing the organism to deal with the stressful situation. Glucocorticoids also regulate the magnitude and duration of the HPA response by way of negative feedback at both the level of the pituitary and hypothalamus, inhibiting the secretion of CRH and consequently ACTH. Image modified from (Dedic et al., 2018).

1.6.4 Emerging role for direct CRH signalling systems unrelated to the HPA-axis

Many neuropeptide hormones, historically considered to be restricted to the brain and pituitary, are now known to be widely expressed in peripheral tissues. The broad distribution of CRH receptors and expression of CRH/UCNs in the periphery suggest functions for these biologically

active peptides other than as hypothalamic neuropeptides. Biological actions of CRH peptides outside of the CNS have largely been derived from experimental studies where the peptides have been applied to cells/tissues *in vitro* or administered *in vivo.* Administration of physiological or pharmacological doses of exogenous CRH or urocortins reveals the ability of these signals to influence a range of physiological and potentially pathophysiological responses. Under normal circumstances, plasma levels of CRH are low. Early reports have suggested circulating CRH and UCN1 in humans in the picomolar range (Sasaki et al., 1987; Watanabe et al., 1999).

Expression of CRH-like peptides in peripheral tissues displays considerable overlap with the tissue distribution of CRHRs suggesting that the ligands may be produced and released locally within tissues to exert classical autocrine and paracrine actions as well as possible endocrine roles (Boorse and Denver, 2006; Grammatopoulos, 2008). A recent review by Squillacioti and colleagues has described the diverse biological roles of the urocortin neuropeptides in regulating homeostatic mechanisms, particularly in mammalian endocrine systems, including the gastrointestinal tract, reproductive organs and the pancreas (Squillacioti et al., 2019). The CRH family has been reported to have a direct immunomodulatory role as mediators of inflammation within both gastrointestinal and pregnancy physiology as well as a regulatory role in energy homeostasis acting locally within key metabolic tissues (Chatoo et al., 2018; Kuperman and Chen, 2008; You et al., 2014). Consequently, many more studies are now underway in attempt to elucidate novel roles for the CRH system in other peripheral tissues and physiological processes.

1.6.4.1 Role of CRH/UCNs in pregnancy

It has been over two decades since CRH immunoreactivity was first reported in human placenta (Rosen et al., 2015; Shibasaki et al., 1982). Subsequent studies have confirmed that the source of maternal plasma CRH during pregnancy is the placenta with levels increasing exponentially as pregnancy advances and peaking at term (Grino et al., 1987a; Thomson, 2013). This has led to the suggestion that CRH may act as a 'placental clock', determining the length of pregnancy. Placental CRH generally acts in a paracrine or endocrine fashion, secreted into both maternal and fetal circulations (Stalla et al., 1989) and thus has the capacity to exert profound effects on maternal and fetal physiology. One of the crucial maternal adaptations CRH is able to influence is the transition of uterine quiescence to coordinated contractions and thus progression of gestation to parturition (Power and Schulkin, 2006). Although the cascade of physiological events mediating this are still incomplete, it has been proposed that direct signalling of CRH (through CRHR1) can promote a cascade of inflammation in the uterus and regulate differential calcium signalling in non-labouring and labouring myometrial tissues (You et al., 2014, 2012).

Intriguingly, placental CRH production was thought to be restricted to anthropoid primates as studies by Robinson and colleagues in the 1980's did not detect CRH mRNA in rat, guniea pig

and lemur placentae. Thus it was concluded that the CRH system is absent from placental mechanisms controlling pregnancy and labour in rodents (Robinson et al., 1989). Several studies have now revealed that not only do human gestational tissues (such as trophoblasts and fetal membranes) express CRH, UCNs and CRHRs but similarly, mice express mRNA for all four peptides and both CRHRs in the placenta, decidua and fetal membranes (Drynda et al., 2018; Petraglia et al., 2010; Voltolini et al., 2015, 2012). In fact, mouse placental UCN2 mRNA and protein expression increases during gestation suggesting UCN2 contributes to pregnancy physiology (Voltolini et al., 2015). Nonetheless, most studies investigating the influence of the CRH system during pregnancy have focused on the role of CRH (particularly in humans) and therefore, limited characterisation of the role of UCNs in either rodent or human pregnancy exists.

1.6.4.2 Role of CRH/UCNs in the endocrine pancreas

The pancreatic islets have recently emerged as a site of local CRHR signalling suggesting a role for the CRH peptide ligands in islet function and/or hormone secretion. As early as 1983, CRH immunoreactivity had been reported in most vertebrae endocrine pancreata with Schally's group detailing the majority of CRH positive cells in the rat and the mouse located at the periphery of the islets of Langerhans compared to a more fluid distribution over the entire islet tissue in the human, monkey and cat pancreas (Petrusz et al., 1983). Functional studies in isolated pancreatic islets and perfused rat pancreas conducted by Moltz soon after CRH was detected in the endocrine pancreas highlighted the effects of CRH to influence islet glucagon and insulin secretion (Moltz and Fawcett, 1985a, 1985b). In recent years, expression of other members of the CRH family (i.e. UCN3) have been reported more specifically in pancreatic β-cells using mouse and rat β-cell lines (Li et al., 2003). Gene expression studies and immunohistochemistry have also been valuable in providing evidence to support a tissue specific CRH receptor system within the endocrine pancreas. Expression of CRHR1 and CRHR2 has been confirmed in rodent and human islets although CRHR1 levels are notably higher than levels of CRHR2 (Amisten et al., 2013; Kanno et al., 1999; Schmid et al., 2011).

There is now increasing evidence that the CRH peptide family may be involved in peripheral metabolic control via direct actions on insulin secreting β-cells (Li et al., 2007; Schmid et al., 2011). Despite the earlier studies, exogenous CRH has been demonstrated to potentiate glucose induced, but not basal insulin secretion from primary mouse and human islets (Huising et al., 2010). In the same study, researchers suggested that this was a consequence of CRHR1-induced activation of intracellular cAMP signalling pathways demonstrating that stimulation of MIN6 cells with increasing doses of CRH resulted in accumulation of the second messenger (Huising et al., 2010). The development of synthetic CRHR selective agonists and antagonists have also been valuable in elucidating functional characteristics of CRHR signalling within a wide range of tissues. INS-1 cells cultured with CRH resulted in a significant reduction of apoptosis (as

measured by caspase 3/7 activity) compared to untreated cells. Moreover, CRH had a significant and dose-dependent effect on proliferation of INS-1 cells as revealed by bromodeoxyuridine (BrdU) incorporation, enhancing proliferation by ~40% after 24 h culture. These effects were partly abrogated with Astressin (a non-selective CRHR antagonist) (Schmid et al., 2011).

Consistent with the positive effects of CRH on islet function *in vitro*, administration of CRHR peptide agonists *in vivo* to mice demonstrated that specific CRHR1 activation resulted in significant increases in plasma insulin in response to glucose challenge in treated animals compared to vehicle controls. Moreover, these animals also displayed improved glucose tolerance compared to vehicle-treated controls (Huising et al., 2010). β-cell- derived UCN3, which is strongly expressed in mammalian pancreatic β-cells, can also stimulate insulin and glucagon secretion particularly in the presence of nutrient excess (Li et al., 2007, 2003).

Although increasing evidence has demonstrated a functional CRH/CRHR system in rodent islets, there is limited data exploring this system locally within human pancreatic islets. Thus, only two independent studies have either reported expression of CRH and CRHR1 mRNA in primary human islets or the effects of ovine CRF to potentiate GSIS from human islets *in vitro* (Huising et al., 2010; Schmid et al., 2011). Though it has been postulated that CRH/CRHR signalling within the islet may perhaps coordinate the activity of the counterregulatory hormones and paracrine regulation of insulin secretion, this has not been established (Huising, 2020). However, studies exploring the transcriptional landscape of mouse β-cells compared to human β-cells have highlighted key differences in gene expression of CRH ligands. Human α-cells have thus been shown to robustly express CRH and whereas UCN3 is highly and selectively expressed in mouse β-cells, expression of the peptide is a common feature of both human α- and β-cells (Benner et al., 2014). Data currently does not exist on the expression or functional effects of the remaining peptides, UCN1 and UCN2. However, the distinct expression patterns of CRH ligands among islet cells between humans and rodents may suggest differences in the potential regulatory effects of the peptides on hormone secretion. Future functional studies in human islets are thus necessary to investigate this possibility and how the findings from mouse studies are applicable to humans.

Despite the data supporting the direct effects of CRHR signalling on islet function, the physiological relevance of this interaction is unclear. As has been discussed above, there is some evidence that placentally derived CRH and urocortins are involved in various biological functions associated with pregnancy. Given that circulating levels rise (for CRH in human pregnancy) and the CRH family of ligands are expressed by the placenta, coupled with the fact that islet cells express both CRHRs, pregnancy may represent a physiological state in which the CRH family may play a role in regulating islet function.

1.7 Aims

Although emerging data has begun to uncover a potential role for the CRH family within pancreatic islets that is independent of the HPA-axis, the physiological relevance for islet cells responding to endogenous CRH and urocortins is still largely unknown. Most of the current literature exploring peripheral CRH signalling proposes a local autocrine and/or paracrine function in respective tissues which appear to support many of the adaptive responses to stress or potential alterations to normal homeostatic mechanisms. However, endocrine signalling to distal CRH/urocortin sensitive tissues may also exist. It is generally accepted that circulating levels of CRH is low in most species however experimental studies conducted within the group have revealed upregulation of mRNA expression of CRH and urocortin peptides in the mouse placental secretome supporting the idea that these peptides could serve as endocrine mediators within the systemic circulation during pregnancy. The increasing evidence that the CRH peptide family may be involved in peripheral metabolic control via direct actions on insulin secreting β-cells additionally suggests that these peptides may be able to influence adaptive responses of β-cells induced during pregnancy. Therefore, the primary aim of this project was to investigate whether there is a physiological role for the CRH system in the pancreatic islet adaptations to pregnancy.

Hypothesis: Placental-derived CRH and urocortin peptides modulate islet specific CRHR signalling involved in pancreatic islet adaptations during pregnancy.

An outline of the individual project objectives are as follows:

- To confirm and characterise islet CRH receptor profile and the effects of CRH receptor (CRHR) activation on islet function *in vitro* using isolated male and female mouse islets.
- To elucidate the islet CRHR and circulating ligand profile during pregnancy and assess the physiological consequence of chronically blocking the effects of endogenous CRH and urocortin ligands on β-cell adaptations and glucose homeostasis during pregnancy.
- To investigate the effects of exogenous CRH ligands on glucose tolerance in an alternative animal model of impaired glucose homeostasis
- To investigate potential correlations between CRH and urocortin peptide levels and glucose intolerance in pregnant women using an archive of pregnant plasma samples.

Chapter 2

Chapter 2 Materials and Methods

This chapter describes the general materials and methods employed throughout this project. Chapter-specific methods and any further experimental details will also be described in the relevant results chapters.

2.1 Animals

Experimental animals such as mice and rats are a valuable resource used in scientific research. Identifying the most appropriate animal model to use is an important consideration to factor into experimental design. Inbred strains and outbred stocks represent the two major classes of available laboratory rodents. Inbred strains (e.g. C57BL/6 mice) are derived from 20 or more consecutive generational sib-matings (brother-sister matings) making animals from the same inbred strain effectively genetically identical. Therefore, it is possible to eliminate genetic variability as a complicating factor when comparing results from independent experiments. However, many disease phenotypes often have complex genetic influences and therefore the use of inbred strains may impede a complete understanding of disease pathophysiology. Additionally, inbred strains may cost as much as 3-4 times more than an outbred stock (Festing, 1979). Outbred stocks (e.g. CD1 mice) are maintained by random (or haphazard) mating and thus each animal is genetically different. Therefore, outbred stocks are considered to most likely reflect the genetic heterogeneity in the human population. CD1 mice are widely used as a research model as they are vigorous, have good breeding qualities (tend to produce more pups per litter than any inbred mouse strain) and are relatively cheap to purchase (Chia et al., 2005). Given these characteristics, they are often the standard wildtype model of choice for experiments.

Outbred CD1 mice and inbred C57BL/6 and obese, leptin deficient ob/ob mice were utilised throughout this project. More discussion on the phenotype of ob/ob mice is presented in Chapter 5 however, as the background of these mice is on the C57BL/6 mouse strain, C57BL/6 mice served as lean controls for corresponding *in vivo* studies. Both mouse strains/stock exhibit good insulin secretion profiles *in vitro* however, CD1 mice were used for islet isolation and functional *in vitro* studies as a readily available stock of these mice were accessible and these mice are slightly larger and therefore a higher yield of islets is typically achieved (Marzorati and Ramirez-Dominguez, 2015). CD1 mice also exhibit very reliable reproductive and good maternal characteristics. As CRHR signalling was characterised using islets from this stock, *in vivo* pregnancy studies using osmotic minipumps were carried out in CD1 mice also. C57BL/6 mice have proved to be useful pathophysiological models for *in vivo* studies of diabetes. Genetic obesity in C57BL/6 as modelled by the ob/ob mouse strain, display severe insulin resistance but do not develop diabetes. The moderate hyperglycaemia in these mice make them useful models

to study potential therapeutic interventions. Therefore C57BL/6 and ob/ob mice were used for later *in vivo* osmotic minipump studies to investigate the potential therapeutic efficacy of UCN2. It is important to note however, that differences in glucose metabolism exists between CD1 and C57BL/6 mice with several studies providing evidence that C57BL/6 mice have increased susceptibility to hyperglycaemia/diabetes and thus are generally less glucose tolerant than CD1 mice (Bowe et al., 2014; Kaku et al., 1988). Therefore, it is possible that more pronounced metabolic phenotypes may be seen in C57BL/6 mice or strains of mice on this background. Nevertheless, it is also important to achieve a reproducible effect in various strains of mice, especially if, as is the case with the ob/ob mice, that a single gene mutation is responsible for the phenotype of the mice, which as mentioned above, doesn't necessarily reflect the aetiology of many multifactorial diseases. Caution when generalising results between strains and understanding the limitations of each animal model was taken into account throughout the thesis.

2.1.1 General overview of study design

All studies involving regulated procedures on research animals were conducted with approval from the King's College London Animal Welfare and Ethical Review Board and were undertaken in accordance with the United Kingdom Home Office Animals (Scientific Procedures) Act 1986. Mice were housed under controlled conditions (12-hour light (0700-1900) /dark cycle, temperature 22 ± 2ºC) and provided with standard chow diet and water *ad libitum*. Animals were typically housed between 2-5 (same sex and strain) per cage, except during mating for timed pregnancy studies.

All experimental mice were used between 8 – 16 weeks of age. Male or female CD1 mice (Charles River Laboratories, Harlow, UK) were used for islet isolation experiments and *in vitro* studies. Osmotic minipump studies utilised both female (pregnant or non-pregnant) CD1 mice and male C57BL/6 and ob/ob mice (Envigo Laboratories, Bicester, UK). Administration of CRH-like peptides (i.e. UCN2) were investigated *in vivo* in all strains of mice. Further details of the experimental animals used for specific experiments are provided in the relevant chapter methods sections.

2.2 Isolation of mouse islets

In vitro tissue culture models of β-cell lines (e.g. INS-1 or MIN6) represent useful, stable and inexpensive tools for studying specific β-cell function and cellular mechanisms as a surrogate for primary tissue. However, these cell lines do not fully represent the physiological behaviour and dynamic interaction of endocrine cells occurring *in vivo*. Potential differences in glucose responsiveness and the reduction in magnitude of response and threshold of glucose response with time in culture, makes cell lines a sub-optimal alternative to complement actual responses *in vivo*

(Hohmeier and Newgard, 2004; Neuman et al., 2014). Therefore, islets isolated directly from mouse pancreatic tissue provides a more precise understanding of how the islet is responding to specific stimuli whilst maintaining morphological and functional characteristics as they do *in vivo*.

Isolation of pancreatic islets was first reported by Bensley in 1911 (Bensley, 1911) and has provided a valuable technical achievement for advances in islet biology research as well as clinical islet transplantation. Since the birth of the technique several researchers have modified the procedure to increase the yield and quality of islets obtained. Dr Moskalewski was the first to introduce the use of collagenase in islet isolation in 1965 (Moskalewski, 1965) however, the collagenase method was perfected in rodents by Gotoh et al in 1985 (Gotoh et al., 1985). These researchers performed intra-ductal injection of collagenase into the common bile duct instead of the previously used cold saline buffer. Islet isolation procedures were further optimised with the adoption of the use of density gradients and filtration steps to improve the islet purification process from acinar tissue (Ramírez-Domínguez, 2016). Isolated islets can be maintained as viable units in culture to be used in acute experiments to monitor islet function or preserved so that other downstream applications (e.g. RNA isolation and real time quantitative polymerase chain reaction (qPCR)) may be pursued. Primary mouse islets were isolated as per the below described protocol for the utilisation in subsequent functional *in vitro* assays.

Mouse islets were isolated via pancreatic collagenase digestion using collagenase Type XI (1 mg/ ml, Sigma, UK) dissolved in Minimum Essential Media (MEM) (Sigma, UK) followed by a Histopaque-1077 (density: 1.077 g/ ml; Sigma, UK) density gradient (as previously described (Carter et al., 2009; Gotoh et al., 1985)). Animals were euthanised by either cervical dislocation or overdose of pentobarbital sodium (1g/ kg) (Euthatal®, Merial Animal Health Ltd, UK).

A midline incision was made in the abdominal cavity using mayo surgical scissors. The posterior sternum cartilage was excised allowing for the liver to be flipped over the chest and secured using a disposable tissue, thus exposing the junction of the gall bladder and common bile duct. A bulldog clamp was used to clamp the Vater's ampulla, the junction where the bile and pancreatic ducts empty into the duodenum. The common bile duct was cannulated with a 27G needle (secured to a 2.5 ml syringe) and 2.5 ml of collagenase solution (1 mg/ ml) injected into the duct to inflate the pancreas (as illustrated in Figure 2-1). The pancreas was then carefully excised using forceps, detaching the tissue from surrounding connective tissue and placed in a 50 ml falcon tube on ice.

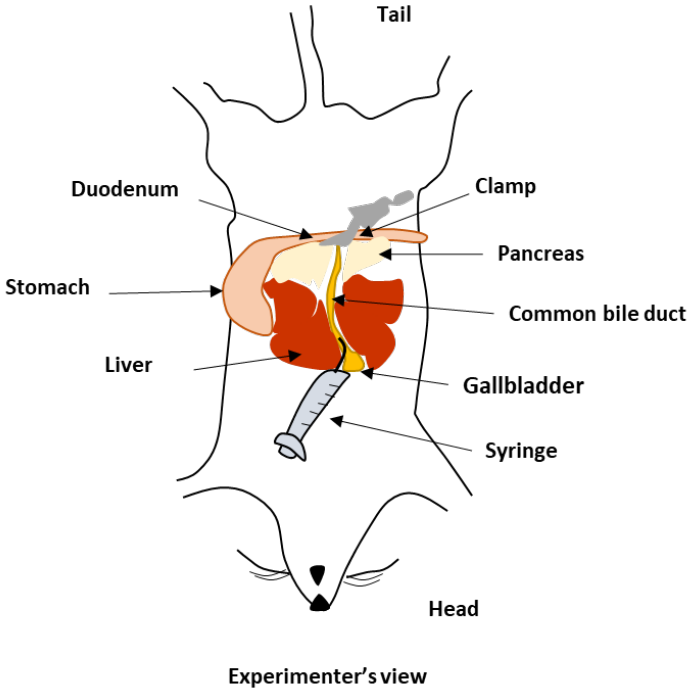

Figure 2-1 Illustration of the mouse pancreatic islet isolation procedure by collagenase digestion. Image adapted from (Carter et al., 2009).

Subsequent enzymatic digestion of the pancreatic exocrine tissue was achieved by incubating the isolated pancreatic tissue at 37^0C for 10 minutes. Collagenase activity was then inhibited by the addition of 25 ml of MEM (wash buffer) supplemented with 10% (v/v) newborn calf serum (NCS) (Sigma, UK) and 1% (v/v) penicillin/streptomycin (100 U/ ml penicillin, 0.1 mg/ ml streptomycin) (Sigma, UK). The pancreas digest was further disrupted, shaking by hand for 10 seconds to completely separate the tissue. Following this, the homogenate was centrifuged at 300 *g* for 1.5-2 mins at 10ºC. This wash process was completed 3 times to ensure removal of collagenase with the supernatant discarded after each wash. Pancreas digests were then resuspended in 25 ml of MEM and filtered through a 425 µm stainless steel sieve (to remove any large undigested material) into a new 50 ml falcon tube. Following centrifugation (400 *g* for 1 min 30 secs, 10ºC) the supernatant was discarded and the pellets drained upside down to remove any excess media. The pellets were then resuspended in 15 ml of Histopaque-1077 (Sigma, UK) before the slow addition of 10 ml of wash buffer (supplemented MEM as above) to create a density gradient, allowing for the purification of islets. Tubes were then centrifuged (1,200 *g* for 25 mins at 10ºC) with slow acceleration and no brake.

Following centrifugation, islets separated from the pancreatic exocrine tissue were located at the Histopaque - MEM interface and were recovered using an automated pipette. The islets were washed a further 3 times with 50 ml fresh MEM (wash buffer) with centrifugation at 400 *g* for 1 min 30 secs, 10ºC. On the final wash, 15 ml of islet suspension was transferred into sterile 90 mm

suspension culture dish and islets subsequently hand-picked using a dissecting microscope. Islets were sterile washed twice in islet culture media- Roswell Park Memorial Institute (RPMI)-1640 (Sigma, UK), supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) L-glutamine and 1% (v/v) penicillin/streptomycin (100 U/ ml penicillin, 0.1 mg/ ml streptomycin) (Sigma, UK). Islets were maintained in culture in a humidified incubator at 37ºC (95% air, 5% CO_2) for a minimum of 24 hours prior to *in vitro* assays.

2.3 Assessment of islet function *in vitro*

To investigate the effects of CRHR activation on islet function, such as their insulin secretory capacity and insulin content, static incubation or dynamic perifusion assays were performed. Both assays are reliable methods to determine whether substances can provoke insulin release in response to glucose stimulation. The static measurement of insulin secretion is able to identify compounds that can initiate or potentiate insulin secretion and provides researchers an opportunity to examine numerous concentrations and conditions from the same batch of islets (Nolan and O'Dowd, 2009). Dynamic perifusion experiments require more islets and are more complex to set up, and as such are not suitable for testing a wide range of treatments and variables. However, perifusion experiments enable the measurement of the timed kinetics of insulin secretion i.e., whether the stimulation of insulin secretion is acute or continuous for the duration of islet treatment and allows for the biphasic insulin secretory response to be observed in greater detail. In both assays, it is necessary to use a balanced salt solution/buffer to provide a physiological isotonic environment and ensure suitable islet functionality. For example, Gey & Gey or Kreb's Ringer bicarbonate buffer can be used (Nolan and O'Dowd, 2009). Both buffers have a similar salt composition though the relative concentrations of each salt in these buffers may slightly differ. As such, despite the evidence of the importance of Ca^{2+}, K^+ and Na^+ (which are routinely supplemented in these buffers) in the regulation of insulin secretory responses, several studies have now reported that changes in the ionic composition of the incubation medium can influence insulin secretion (Hales and Milner, 1968; Henquin and Lambert, 1975; Milner and Hales, 1967). Therefore, it is possible that slight variations in the secretory responsiveness may be observed between studies which use either buffer. Hence it is important to bear this in mind when making comparisons to similar studies within the literature.

2.3.1 Static incubation

To investigate the effects of CRHR activation on glucose-induced insulin secretion, isolated islets were incubated either in the presence or absence of CRHR specific agonists or antagonists at sub-stimulatory (2 mM) or stimulatory (20 mM) glucose concentrations. All islets used for static incubations were allowed to recover for a minimum of 24 h following isolation.

Prior to the static incubation experiment, islet culture medium was replaced with a physiological salt buffer (Gey & Gey)(G&G) (Gey and Gey, 1936) (Table 2-4) supplemented with 2 mM glucose (Table 2-1 for working solution) and left to incubate in a humidified incubator (37ºC, 95% air, 5% CO_2) for 1 h to achieve basal insulin secretion. Following this pre-incubation period, groups of 5 size-matched islets were hand-picked on ice into 1.5 ml Eppendorf tubes containing 500 µl of 2 mM or 20 mM glucose concentrations in G&G buffer, supplemented with or without agonist/antagonist (summarised in Table 2-2). Each treatment condition consisted of between 6-10 replicates. Tubes were subsequently placed in polystyrene floatation racks and placed in a 37ºC water bath for 1 h. Following incubation, tubes were centrifuged (150 *g* for 2 mins at 4ºC) before collecting 300 µl of supernatant. Samples were stored at -20ºC for later insulin quantification by radioimmunoassay (section 2.3.4).

2 mM glucose Gey & Gey buffer working solution

Reagent	Amount	Final concentration
Gey & Gey buffer [2x stock] (Table 2-4)	250 ml	1 x Gey & Gey
Distilled water	250 ml	-
D-glucose (Sigma, UK)	180 mg	2 mM
1M Calcium chloride (CaCl2) (Honeywell, Germany)	1 ml	2 mM
Bovine Serum Albumin (BSA) (Sigma, UK)	250 mg	0.5 mg / ml

Table 2-1 Composition of Gey and Gey buffer working solution for 2 mM glucose. pH of the buffer was adjusted to 7.4 using 95% air and 5% CO_2 prior to the addition of Bovine Serum Albumin (BSA). To make 20 mM glucose working solution, 810 mg of D-glucose was added to 250 ml of 2 mM glucose Gey & Gey buffer.

2.3.2 Dynamic perifusion

A temperature - controlled (37ºC) room housing perifusion apparatus (as show in Figure 2-2) was used to perform dynamic perifusion insulin secretion experiments using isolated mouse islets (as previously described (Persaud et al., 2002)). The apparatus consists of a water bath (set at 37ºC) where buffer reservoirs are maintained. The buffer reservoirs are connected to a series of small, individual chambers via 2 mm diameter tubing, joined to multiple valves which control transfer of various buffers to the islet chambers. The distal tubing connects the chambers to a peristaltic pump which delivers the respective buffers to the islet chambers and allows the collection of subsequent secreted islet perifusate into a 96-well block at a rate of 0.5 ml/ min.

The dynamic insulin secretory profile of islets following acute or chronic (48 h) exposure to various CRHR agonists (summarised in Table 2-2) on glucose-stimulated insulin secretion was

investigated in the perifusion system. Pre-warmed buffers were flushed through the system to remove any air bubbles that could be exposed to the islets. The final buffer used to flush the system was 2 mM glucose G&G as this buffer was used to pre-incubate islets for a minimum of 1 h to achieve basal insulin secretion. Hydrophilic, nylon mesh (pore size -1 μm, Millipore, UK-cut according to chamber diameter) was pre-soaked in 2 mM glucose G&G prior to positioning inside the chamber. This nylon mesh allows buffer flow through the chamber but prevents islet outflow into the system. Groups of 35 – 40 islets were hand-picked into each chamber using a pipette and the perifusion system was allowed to run for 1 h with G&G supplemented with 2 mM glucose, preincubating the islets at basal insulin release.

Following preincubation, experiments commenced, and islets were exposed to buffers containing various treatments at specific time-intervals. Secreted fractions were collected every 2 minutes into a 96-well block. Sample fractions were stored at -20ºC until later insulin quantification by radioimmunoassay (described in section 2.3.4).

CRHR agonist	**CRHR target**	**Concentration**	**Supplier**
CRH	CRHR1/ CRHR2	50 nM	Sigma, UK [Catalog # C3042]
UCN2	CRHR2	100 nM	Sigma, UK [Catalog # U9507]
Stressin I	CRHR1	100 nM	Tocris, UK [Catalog # 1608]
CRHR antagonist	**CRHR target**	**Concentration**	**Supplier**
Antalarmin hydrochloride	CRHR1	1μM	Tocris, UK [Catalog # 2778]
Astressin 2B	CRHR2	1μM	Tocris, UK [Catalog # 2391]

Table 2-2 Summary of CRH receptor (CRHR) agonists/antagonists used in either static incubation or dynamic insulin secretion studies.

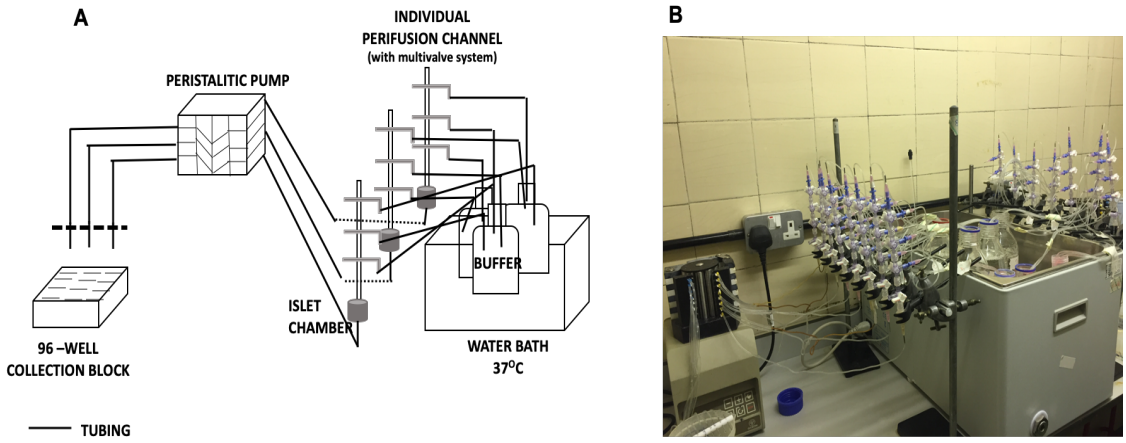

Figure 2-2 Diagram (A) and image (B) of in-house, temperature -controlled (37°C) dynamic perifusion apparatus. Islets (35-40) were loaded into respective islet chambers and perifused with buffers of interest (flow rate 0.5 ml/ min) with the resulting perifusate sample collected into a 96-well block at 2-minute intervals.

2.3.3 Insulin content

Measurement of pancreatic islet insulin content was performed following chronic (48 h) incubation of islets in islet RPMI culture medium (described in section 2.2) supplemented with 50 nM CRH (Sigma, UK) or 1 µM α-helical CRF_{9-41} (Tocris, UK). Following incubation, 10 islets were hand-picked using a pipette and lysed in 50 µl of acidified ethanol (absolute ethanol: distilled water: concentrated HCl in the ratio of 52:17:1). Samples were sonicated (Soniprep 150) on ice for 3 x 5 second pulses at an amplitude of between 10- 14 KHz. Samples were subsequently stored at -20°C until insulin quantification by radioimmunoassay (described below).

2.3.4 I^{125}- Insulin Radioimmunoassay

Radioimmunoassay (RIA) is a sensitive method for determining concentrations of an antigen in a given sample and enables large numbers of samples to be assayed with good precision (Goldsmith, 1975). RIA utilises radioactively-labelled antigens (i.e. the tracer) which competes with unlabelled antigens (i.e. in the sample) for limited, specific antibody binding sites, consequently forming antigen-antibody complexes as demonstrated in Figure 2-3. By preparing a standard curve with known concentrations of non-radiolabelled antigen and fixed amounts of radiolabelled antigen and the specific antibody, unknown sample quantifications can be determined. This is because the ratio of antigen-antibody complexes formed at equilibrium is

dependent on the displacement of tracer-bound antibody by increasing amounts of unlabelled antigen in solution and vice versa. Therefore, when measuring the specific radiation emitted in counts per minute (cpm), an inverse relationship is exhibited between the amount of tracer and antigen in the reaction (i.e. more tracer-antibody complexes, and thus higher cpm indicates lower amount of unlabelled antigen in sample).

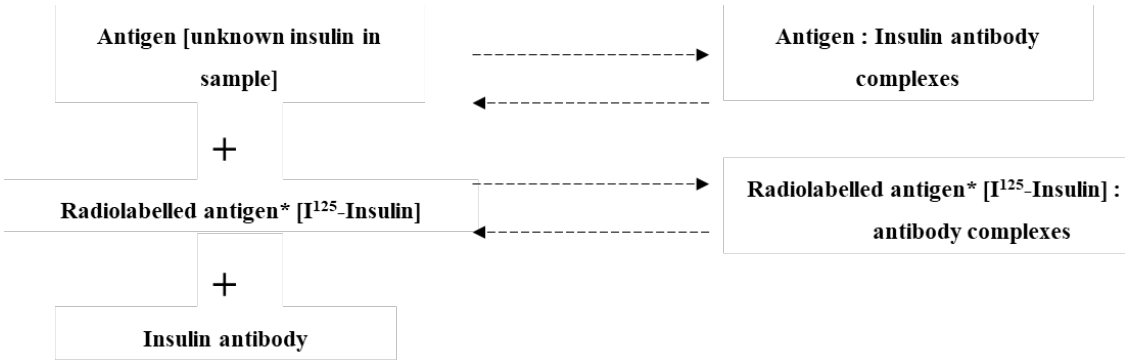

Figure 2-3 Equation demonstrating the reaction occurring in the I^{125}-Insulin radioimmunoassay. The addition of an unknown concentration of antigen (i.e. insulin) in each sample with radiolabelled antigen (I^{125}-Insulin -> tracer) and a fixed concentration of specific antibody (Insulin antibody) will subsequently result in the above detailed antigen complexes being formed at equilibrium. The amount of radiolabelled antigen: antibody complexes can be measured using a γ-counter. The standard curve prepared within the assay can then be used to interpolate quantities of antigen within unknown samples.

2.3.4.1 Protocol

An in-house I^{125}-Insulin radioimmunoassay was used to quantify unknown insulin-containing samples as previously described (Jones et al., 1988). A standard curve ranging from 0.04 – 10 ng/ ml was prepared in triplicate from the serial dilution (1:2) of a 10 ng/ ml purified rat insulin stock diluted with borate buffer (see composition of buffer in Table 2-5). A series of reference tubes; Maximum binding (binding of tracer in the absence of unlabelled insulin), Total binding (total counts per minute of radiolabelled tracer only) and Non-specific binding (binding of tracer in the absence of antibody), were also prepared in triplicate. Experimental samples were either assayed neat or diluted in borate buffer (to allow for the detection limits of the radioimmunoassay) to create a total volume of 100 µl and assayed in duplicate. Guinea-pig anti-bovine insulin antibody was diluted in borate buffer (1:10) to give a final concentration of 1:60,000. Radioactive I^{125}-Insulin (emitting γ radiation) was diluted with borate buffer to achieve an approximate count

of 10,000 cpm per tube. Quantities of assay components are outlined in Table 2-3. Samples were incubated alongside the standard curve at 4ºC for 48 h for the reaction to reach equilibrium.

Following the equilibration period, 1 ml of precipitation solution (see Table 2-8) was added to each tube (except the Totals reference tube) and centrifuged at 1,500 *g* for 15 minutes at 4ºC. The supernatant (containing free antigen and free radiolabelled antigen) was subsequently aspirated and the radioactivity of the resultant pellet (containing antigen: antibody complexes) measured using a γ-counter (Packard Cobra II, PerkinElmer, USA) in cpm per ml. Insulin concentrations in samples were interpolated from the log-transformed standard curve (Figure 2-4 for example standard curve).

	Borate Buffer (µl)	**Insulin Antibody (µl)**	**I^{125}-Insulin Tracer (µl)**	**Insulin standard (µl)**	**Unknown samples (µl)**
Total radioactivity (T)			100		
Maximum binding (B_0)	100	100	100		
Non-specific binding (NSB)	200		100		
Insulin standards		100	100	100	
Unknown samples		100	100		100

Table 2-3 Preparation of the standard curve, reference tubes and samples for insulin radioimmunoassay.

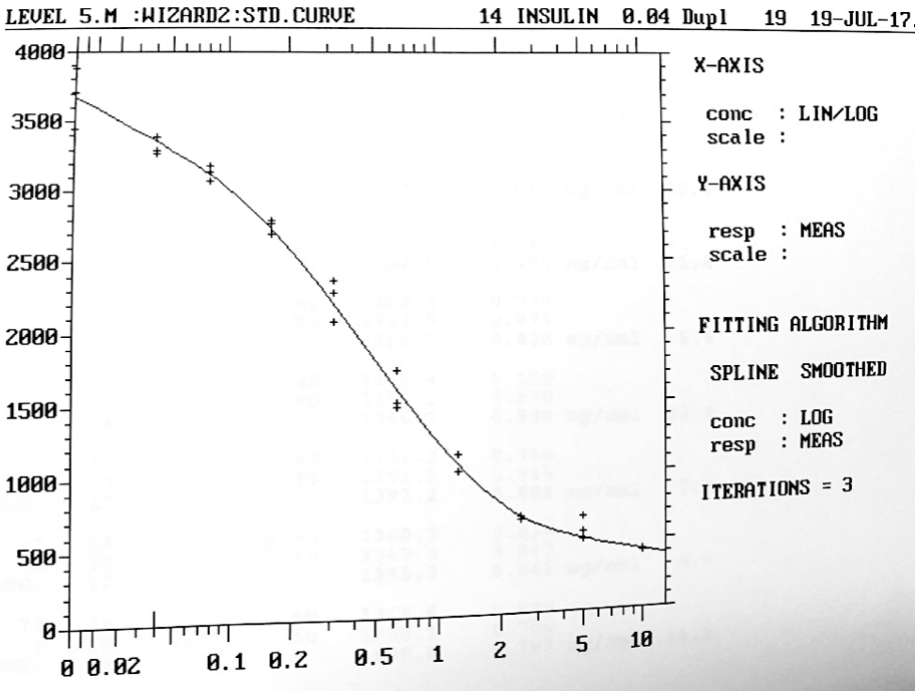

Figure 2-4 Standard curve produced from insulin radioimmunoassay.

2.3.5 Buffer composition for insulin assays and measurement

The summary tables below show the composition of the various buffers used in the insulin secretion assays (static incubation and dynamic perifusion) and the in-house insulin radioimmunoassay used for the quantification of insulin.

Gey & Gey

Reagents		**Amount (g/ 2l) [2x stock]**	**Final concentration when diluted (mM)**
NaCl	Sodium chloride	26.00	111.00
KCl	Potassium chloride	1.48	5.00
$NaHCO_3$	Sodium hydrogen carbonate	9.08	27.00
$MgCl_2.6H_2O$	Magnesium chloride hexahydrate	0.84	1.00
KH_2PO_4	Potassium dihydrogen orthophosphate	0.12	0.22
$MgSO_4.7H_2O$	Magnesium sulphate heptahydrate	0.28	0.28

Table 2-4 Composition of physiological salt solution, Gey and Gey buffer used for insulin secretion assays. Reagents were completely dissolved in 1.5l of distilled water before making the final volume up to 2l with the addition of distilled water, adjusting the pH to 8.4 and stored at 4ºC.

Borate Buffer

Reagents		Amount (g/ 2l)	Final concentration when diluted (mM)
H_3BO_3	Boric acid	16.50	133.00
$C_{10}H_{16}N_2O_8$	Ethylenediaminetetraacetic acid (EDTA)	7.40	10.00
NaOH	Sodium hydroxide	5.40	67.50

Table 2-5 Composition of borate buffer used in radioimmunoassay protocol to quantify insulin. Reagents were dissolved in 1.8l of distilled water and the pH adjusted to 8.0 with concentrated HCl. The final volume was made up to 2l with the addition of distilled water and finally 2g of bovine serum albumin (BSA) was added before storage at 4ºC.

Phosphate buffered saline (PBS)

Reagents	Amount (g/ 10l)
Dulbecco's phosphate buffered saline	95.50
Sodium azide	3.00

Table 2-6 Composition of Phosphate buffered saline (PBS) used in radioimmunoassay protocol to make precipitation buffer. Reagents were dissolved in 9l of distilled water before making to a final volume of 10l by the addition of more distilled water. The solution was stored at room temperature.

30% PEG

Reagents		Amount (g/ 2l)
$C_{2n}H_{4n+2}O_{n+1}$	Polyethylene glycol (PEG- MW. 6000)	600.00

Table 2-7 Preparation of 30% PEG used to make the precipitation buffer in the radioimmunoassay protocol. Reagents were dissolved in 1l of distilled water before being made up to a final volume of 2l with the addition of more distilled water. Solution was stored at 4ºC.

Precipitation buffer (15% PEG)

Reagents	Amount (1l) [15% PEG]	Final concentration (%)
30% PEG	500 ml	15%
PBS	500 ml	-
γ-globulin	1000 mg	1%

Table 2-8 Preparation of precipitation buffer (15% PEG) used in the radioimmunoassay protocol. The γ-globulin was dissolved in PBS before the addition of 30% PEG solution with continuous mixing. Tween-20 (0.5 µl/ ml) was added to the final buffer to prevent the γ-globulin from sticking to the glass assay tubes.

2.4 Gene expression

Determining the levels of messenger ribonucleic acid (mRNA) transcripts (the intermediary between DNA and protein) in a cell allows researchers to study the effects of various experimental conditions on gene expression. The most commonly used application for gene expression analysis is quantitative polymerase chain reaction (qPCR) described in section 2.4.3. RNA is first isolated and characterized for quantity and integrity. Complementary DNA (cDNA) is subsequently synthesized and then used as a PCR template. Amplification of DNA templates in the PCR reaction (using a fluorescence detection system) allows for the detection and quantification of mRNA transcripts in the original sample.

CRHR gene expression was analysed in isolated male and female islets. To also investigate the CRH family and receptor profile during pregnancy, isolated islets from pregnant and non-pregnant female CD1 mice along with placenta, were harvested to measure mRNA levels of CRH receptors and ligands respectively. RNA was first extracted from frozen samples using the commercially available RNeasy Mini Kit (Qiagen, UK) which utilises silica-based membrane column technology and a high-salt buffer system to purify high quality RNA. A brief description of the protocol is detailed below.

2.4.1 RNA extraction

Approximately 150 – 200 frozen mouse islets or 30 mg of frozen placental tissue was first lysed by the addition of 350 – 600 µl of buffer RLT (reconstituted with 1% β-mercaptoethanol). As this lysis process releases intracellular RNases which rapidly degrade RNA, the buffer RLT, containing a high concentration of guanidine thiocyanate (a potent protein denaturant) in combination with the reducing agent properties of β-mercaptoethanol, ensure the complete inactivation of RNase enzymes. Samples were then homogenised using QIAshredder spin columns for islets (Qiagen, UK) or tissue homogeniser for placenta, vortexed and an equal volume of 70% ethanol transferred to supernatant/samples to provide appropriate RNA binding conditions. Up to 700 µl of lysate was then transferred onto the RNeasy spin column, placed in a 2 ml collection tube and centrifuged (room temperature) for 15 seconds at 13,500 *g*. Contaminants in the sample are removed in the flow through which is discarded, leaving RNA bound to the silica membrane in the column.

A washing buffer containing guanidine salts and ethanol (buffer RW1, 700 µl) was then added to the RNeasy spin column to remove any non-specifically bound biomolecules from the silica membrane. Columns were centrifuged for 15 seconds at 13,500 *g* and again the flow through discarded. An additional washing buffer (buffer RPE, 500 µl) was used to wash the RNeasy spin

column twice more to ensure that any traces of salt were removed, with a short centrifugation (15 seconds, 13,500 *g*) followed by a longer centrifugation (2 minutes, 13,500 *g*) to dry the spin column and ensure no residual ethanol is carried over during RNA elution.

The RNeasy spin column was then placed in a new 1.5 ml collection tube and 30 µl of RNase-free water directly applied to the spin column membrane to elute the RNA. The resultant eluate after centrifugation (1 minute, 13,539 *g*) was subsequently placed on ice and concentration determined immediately by measuring the absorbance at 260 nm using Nanodrop® ND- 1000 spectrophotometer.

2.4.1.1 RNA quantification

As nucleic acids have intrinsic absorptivity properties, absorbing light with a characteristic peak at 260 nm, the concentration of RNA in each sample was quantified immediately after extraction using a spectrophotometer (Nanodrop® ND- 1000, UK). Exposure of sample to ultraviolet light, enables a photodetector to measure the light that passes through the sample which is compared to the light that is absorbed by the sample. The more light absorbed by the sample, the higher the nucleic acid concentration in the sample and thus less light will strike the photodetector, producing a higher optical density.

As an absorbance of 1 unit at 260 nm corresponds to approximately 40 µg RNA/ ml, the concentration of a small sample volume (i.e. 1 µl) can be determined in ng / µl. The purity of RNA samples was also determined by comparing the ratio of absorbance at 260 nm and 280 nm (A_{260}/A_{280}). A ratio between 1.8 - 2.2 is generally accepted as pure RNA. RNA quantification was used to standardise the amount of RNA between samples used for cDNA reverse transcription described below.

2.4.2 Complementary DNA (cDNA) conversion

Purified RNA from mouse islets or placenta was subsequently converted to complementary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems™, UK) and RNA-dependent DNA polymerase (reverse transcriptase). Briefly, RNA levels were normalised to up to 2 µg in a total volume of 20 µl. 10 µl of normalised RNA sample was then added to 10 µl of a constituted 2x reverse transcription (RT) master mix (see Table 2-9), resulting in a total reaction volume of 20 µl. To complete the reverse transcription, samples were then loaded into a thermal cycler (Bio Rad, UK) and the four step program conditions (as detailed in Table 2-9) followed. Samples were then stored at -20ºC until real-time quantitative PCR.

Reagent	Amount (µl)/ single 20 µl reaction			
10x RT buffer	2.0			
25x dNTP mix (100 mM)	0.8			
10x RT random primers	2.0			
Multiscribe^{TM} reverse transcriptase (50 U/ µl)	1.0			
Nuclease-free H_20	4.2			
Thermal cycler settings	**Step 1**	**Step 2**	**Step 3**	**Step 4**
Temperature (^oC)	25	37	85	4
Time (min)	10	120	5	∞

Table 2-9 Preparation of 2x reverse transcription (RT) master mix and Thermal cycler programme settings for cDNA conversion. The High Capacity cDNA RT kit was used to compose RT master mix for a total reaction volume of 20 µl (10 µl of RNA sample + 10 µl RT master mix).

2.4.3 Real time quantitative polymerase chain reaction (qPCR)

In order to robustly detect and quantify gene expression from small amounts of cDNA, amplification of the DNA is performed using polymerase chain reaction (PCR). The reaction incudes a cDNA template, specific primers (for genes of interest), deoxyribonucleosides (dNTPs), a suitable buffer solution and a thermo stable DNA polymerase. Fundamentally the reaction consists of repeated temperature-dependent cycles comprising steps of DNA denaturation, primer annealing and extension. Real time qPCR allows for the collection of data throughout the PCR process as it occurs. Application of fluorescence techniques to PCR methodologies enables the combination of amplification, detection and quantification into a single step hence the term real time qPCR (Ramos-Payán et al., 2003). The PCR is carried out in a thermal cycler which is able to rapidly heat and chill samples thus providing optimum conditions for nucleic acid and DNA polymerase function. The thermal cycler also has the capacity to illuminate each sample with a beam of light to excite the fluorescence detection system added to the PCR master mix and has sensors for measuring the fluorescence during each cycle to generate quantitative results.

The types of fluorescence detection systems commonly employed for real time qPCR include non-specific DNA binding dyes (e.g. SYBR® Green) or sequence-specific DNA probes (e.g. TaqMan). As the synthesis of multiple target-specific probes are required for the latter, incurring high costs, SYBR® Green was utilised for all qPCR experiments in this project. SYBR® Green detects PCR products by binding in the minor groove of double stranded DNA (dsDNA) formed during the PCR. In its unbound state, it has relatively low fluorescence but as more PCR products

are created, SYBR® Green binds to all dsDNA, increasing the fluorescence. Therefore, an increase in fluorescence intensity is proportional to the amount of PCR product produced (Ramos-Payán et al., 2003).

Correction for sample to sample variation in qPCR experiments can be achieved by including a reference gene (also referred to as a housekeeping gene) in the assay. The basic assumption is that a suitable reference gene is expressed in a wide variety of tissues/cell types at a constant level and its transcription is not affected by experimental factors (Rebouças et al., 2013). The reference gene also allows for the relative quantification of target gene transcripts (i.e. a ratio of the expression of the genes of interest divided by the expression of reference gene) meaning that all samples are normalised enabling comparison between samples.

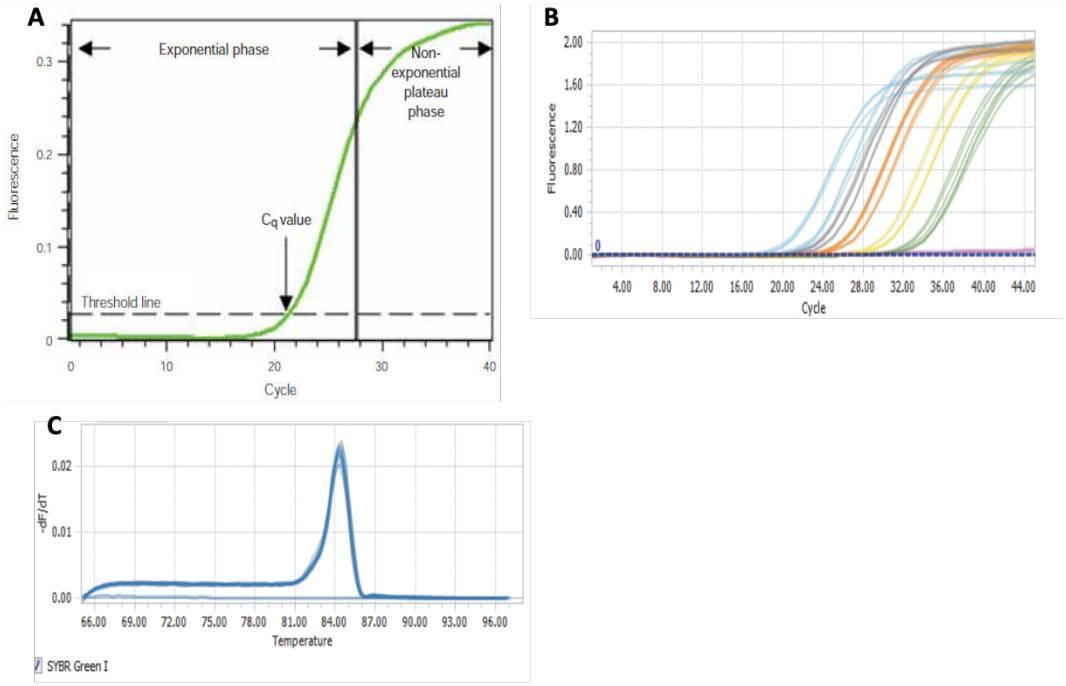

Figure 2-5 Principles of real time quantitative PCR and LightCycler® 96 analysis. Three distinct phases are generally characterised in a PCR amplification curve (A): 1) the linear- ground or baseline phase where fluorescence remains at background levels and increases in fluorescence are not detectable 2) The exponential phase -eventually enough amplified product accumulates to yield a detectable fluorescent signal above the threshold (referred to as the quantification cycle (Cq)) and PCR product approximately doubles in each cycle 3) Finally, the non-exponential plateau phase follows which is reached when reaction components become limited and PCR products/ fluorescence slows. The greater the quantity of target DNA in the starting material, the faster the fluorescent signal passes the threshold, yielding a lower Cq. Amplification curves can be monitored real time in the LightCycler® 96 (B) with each separate curve representing each sample with a specific gene of interest. Analysis of melting curves (C) allows for the verification of product identification as each PCR product will have specific melting temperatures (T_m) and therefore a single melting peak. Any additional peaks could signify non-specific by-products (e.g. primer dimers) that have been amplified in the reaction. Images taken from Bio-Rad website and Roche LightCycler® 96 System Guides.

2.4.3.1 Protocol

Real time quantitative PCR (qPCR) was used to quantify the detection of an amplified target DNA using QuantiTect® SYBR® Green PCR master mix (Qiagen, UK) containing DNA polymerase and bioinformatically validated QuantiTect® oligonucleotide primers purchased from Qiagen

(UK) (see Table 2-10 for master mix & Table 2-11 for primers used). QuantiTect® primers are designed to provide optimal performance with QuantiTect® SYBR® Green assay kits. All real time qPCR reactions were prepared in 96-well plates in duplicate. Following dilution of cDNA (in RNase free H20 to yield a concentration of 15 ng/ µl), 2 µl of this was added to a total volume of 8 µl of SYBR® Green master mix (Table 2-10) (containing the primers of interest as detailed in Table 2-11) to give a total PCR reaction volume of 10 µl. The plate was sealed and then briefly centrifuged for 1 minute (130 *g*, 4ºC) before being placed in the LightCycler® 96 (Roche Diagnostics, UK). Cycler protocol/ conditions are outlined in Table 2-10. The subsequent Cq (quantification cycle) values were determined and the relative expression of genes of interest to the reference gene (i.e. glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*)) was quantified using the LightCycler® 96 application software.

Reagent	**Amount (µl)/ reaction**	
cDNA	2.0	
SYBR® green master mix	5.0	
QuantiTech® primer	2.0	
RNase free H_20	1.0	
LC96 protocol	**Temperature (ºC)**	**Time (sec)**
Pre-incubation	95	300
Amplification	95 60	10 30
Melting	95 65 97	5 60 1
Cooling	40	30

Table 2-10 Composition of SYBR® green PCR master mix and LightCycler® 96 protocol.

QuantiTect® Primers	**Catalogue Number**
Mouse Gapdh	QT01658692
Mouse CRH	QT01055789
Mouse UCN1	QT00326879
Mouse UCN2	QT01556534
Mouse UCN3	QT00302267
Mouse CRHR1	QT00106232
Mouse CRHR2	QT00151543

Table 2-11 QuantiTect® primer catalogue numbers (Quiagen, UK).

2.5 Subcutaneous implantation of osmotic minipumps

The administration of test substances (e.g. peptides, compounds or drugs) to mice is one of the major methods for evaluating their biological activity and/or the physiological consequence of the test agent to the phenotype of the animal model. Several routes of administration exist however, selecting the most appropriate route is largely dependent on the objective of the experiment and the property of the test substance (Hedrich and Bullock, 2004). Longer term studies looking at the chronic effects of test substances warrant the need for a method to continually dose with minimal distress to the animal. Implantable osmotic minipumps (OMPs) provide a reliable and convenient method of continuous drug delivery *in vivo* whilst minimising stress to animals due to frequent injections and handling (Doucette et al., 2000; Hedrich and Bullock, 2004). Osmotic minipumps can be used for systemic administration when implanted subcutaneously or intraperitoneally and are particularly advantageous in studies utilising smaller laboratory animals due to their compact size. The pumps are self-powered through the process of osmotic displacement. The osmotic minipumps consist of 3 concentric layers; a semi-permeable outer membrane, an osmotic layer/salt sleeve and an inner drug reservoir (0.1 -0.2 ml) to which the flow moderator is positioned (see Figure 2-6B). Once the pumps are loaded with test agents to be delivered and implanted, water is absorbed through the outer membrane. This results in the expansion of the osmotic layer which compresses the flexible, impermeable reservoir, enabling the test solution to be released through the flow moderator at a controlled rate for a period of up to two weeks. Osmotic minipumps were employed throughout our *in vivo* studies to chronically administer CRHR antagonists or agonists to mice, to investigate their effects on glucose homeostasis.

2.5.1 Protocol

Osmotic minipumps (OMPs) (ALZET®, Model 1002, Charles River, UK) were used in studies to chronically administer CRHR antagonists to pregnant and non-pregnant CD1 mice or CRHR agonists to C57BL/6 and to ob/ob mice. Prior to scheduled implantation, OMPs were loaded with 100 µl of respective test agents (as summarised in Table 2-12) and then individually immersed in bijou tubes (containing approximately 4 ml of sterile saline) to prime pumps.

Mice were anesthetised with isoflurane (4%) and 96% oxygen (airflow set between 2- 4 l/min) via an induction chamber before shaving a small patch of the dorsal back region. The implantation site was then disinfected with surgical iodine and cleaned with ethanol (70%). Mice were placed in the prone position (lying face down) and maintained on isoflurane (2-3%) using a nose cone and appropriate scavenger. An incision of approximately 1 cm was made using a scalpel, close to the midscapular region and a small pocket under the skin was created by spreading a mosquito

hemostatic forceps cortically at the site of implantation. The pre-loaded osmotic minipump was then inserted subcutaneously, delivery port first, into the pocket and the incision sealed with 2 – 3 resorbable sutures (VICRYL™, Aston Pharma, UK). Animals were left to recover in a heated chamber before being returned to their normal cages and monitored daily. Antagonists or agonists were continuously delivered to mice at an infusion rate of 0.25 µl/ h for a maximum duration of 14 days.

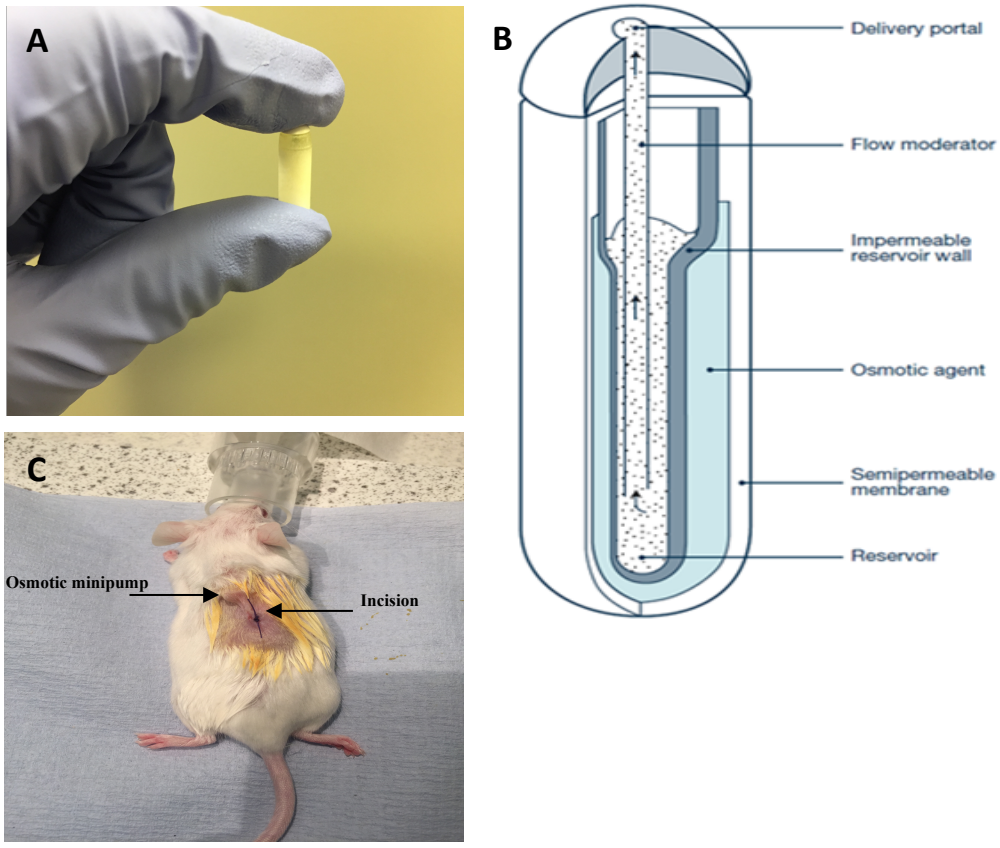

Figure 2-6 Image (A) and schematic of ALZET® osmotic minipump (B) including an image of completed implantation of osmotic minipump into female CD1 mouse (C).

CRHR Antagonist Studies			
Antagonist/ Agonist	**CRHR target**	**Concentration loaded in OMP**	**Supplier**
α-helical CRF_{9-41}	CRHR1/ CRHR2	1 mg/ ml	Tocris, UK [Catalog # 1184]
Antalarmin hydrochloride	CRHR1	1 mg/ ml	Tocris, UK [Catalog # 2778]
Antisauvagine-30	CRHR2	3 mg/ ml	Tocris, UK [Catalog # 2071]
CRHR Agonist Studies			
UCN2	CRHR2	Dose 1: 83.04 µg/ ml Dose 2: 415.2 µg/ ml	Generon, UK [Catalog # CCP1460]

Table 2-12 Summary of CRHR antagonist and agonist details for osmotic minipump infusion studies.

2.6 Testing glucose homeostasis *in vivo*

Commonly used methods for assessing glucose homeostasis *in vivo* in rodents include the glucose tolerance test (GTT) and the insulin tolerance test (ITT). The GTT is a relatively simple method, providing a physiological overview of any changes in glucose tolerance and can be used to investigate β-cell function *in vivo*. A time course measurement of absolute blood glucose levels following glucose challenge can be obtained by a small blood sample (~5 µl) with a larger blood volume required for additional hormone measurements to assess islet function (Bowe et al., 2014). The ITT is technically very similar to the GTT and can be carried out as an approximate measure of insulin resistance. Blood glucose levels are monitored over a time course in response to insulin administration rather than glucose loading. For rodents in particular, ITTs offer an easier and cheaper method of assessing insulin sensitivity compared to the more technically demanding and invasive hyperinsulineamic-euglycaemic clamp procedure (King, 2012). To assess the effects of pharmacologically blocking endogenous CRH and UCNs or conversely, exogenous peptide treatment on glucose clearance and insulin sensitivity, intraperitoneal glucose and insulin tolerance tests were performed respectively.

2.6.1 Intraperitoneal glucose tolerance test (i.p. GTT)

Mice were fasted from 0900 for 6 hours prior to metabolic testing. After weighing and measurement of baseline (0 min) glucose concentrations by small tail prick, glucose (2 g/ kg, Sigma, UK) was administered via intraperitoneal injection using a 30G needle and 1 ml syringe. Blood sampling for the determination of blood glucose levels at 15, 30, 60, 90 and 120 minutes post glucose load were recorded using either Accu-Chek glucose meter (glucose reading range:

0.6 - 33.3 mM) (Roche Diagnostics, UK) or NOVA Statstrip® Xpress meter (glucose reading range: 1.0 - 55.0 mM) (Data Sciences International, USA) and appropriate test strips.

2.6.1.1 Tail vein blood sampling

In addition to measuring blood glucose throughout the course of the GTT, blood sampling for the measurement of fasted (basal) and glucose stimulated insulin secretion was also performed. Prior to sampling, a topical anaesthetic (EMLA cream, AstraZeneca, UK) was applied to the tails of mice and allowed to absorb for 15 – 20 minutes. Following restraint of mice, a superficial incision (approximately 3 cm from the tip of the tail) was carefully made using a scalpel and approximately 50 µl of blood collected (via pipette) into a sterile Eppendorf pre-coated with heparin (5000U / ml). Sampling was performed at baseline (0 min) and 30 minutes after glucose injection. All samples were kept on ice before being centrifuged (1,800 *g*, 20 minutes, 4^0C) with the subsequent plasma collected and stored at -20^{o}C for later quantification using a mouse insulin ELISA (10-1247-01, Mercodia, Sweden) according to the manufacturer's instructions.

2.6.2 Intraperitoneal insulin tolerance test (i.p. ITT)

Mice were fasted from 0900 for 6 hours prior to metabolic testing. After weighing and measurement of baseline glucose concentrations by small tail prick, insulin (0.75 IU/ kg, Sigma, UK) was administered via intraperitoneal injection using a 30G needle and 1 ml syringe. Blood sampling for the determination of blood glucose levels at 15, 30, 45 and 60 minutes post insulin injection were recorded using either Accu-Chek glucose meter (Roche Diagnostics, UK) or NOVA Statstrip® Xpress meter (Data Sciences International, USA) and appropriate test strips.

2.6.3 Bromodeoxyuridine (BrdU) administration *in vivo*

Bromodeoxyuridine (BrdU) is a synthetic thymidine analogue that is commonly used to detect proliferating cells *in vivo*. The synthetic compound is substituted with the nucleoside thymidine as a substrate for replicating DNA with subsequent BrdU labelling detected using anti-BrdU antibodies.

For CRHR antagonist osmotic minipump studies, BrdU (1 mg/ ml, Sigma, UK) was administered in the drinking water from gestational day 14 to day 18 (or equivalent time intervals in non-pregnant mice). The BrdU-containing drinking water was replaced every two days. At the end of the specified study duration, pancreata were dissected and fixed in 4% formaldehyde (Sigma, UK) for 48 h prior to wax embedding and subsequent immunohistochemistry (as described in section 2.7).

2.6.4 Testing glucose homeostasis in response to CRH-like peptides

UCN2 (0.1mg/ kg) (U9507, Sigma, UK) / (CCP1460, Generon, UK) was intraperitoneally injected into male C57BL/6, ob/ob and CD1 mice to assess the acute effects of the peptide on glucose homeostasis. Blood sampling at 0, 15, 30, 45 and 60 minutes by small tail prick to determine blood glucose levels was performed using Accu-Chek glucose meter (Roche Diagnostics, UK) or NOVA Statstrip® Xpress meter (Data Sciences International, USA) and appropriate test strips.

2.6.5 Terminal blood sampling and tissue collection/extraction

Terminal blood samples were collected (from non-pregnant and pregnant CD1 mice) via cardiac puncture for the measurement of circulating CRH-like peptides. Animals were euthanised by intraperitoneal injection of terminal anaesthesia, pentobarbital sodium (1 g/ kg) (Euthatal®, Merial Animal Health Ltd, UK) before being cut through the peritoneal cavity and rib cage to expose the heart. Blood was slowly drawn from the left ventricle using 25G needle and 1 ml syringe into Eppendorf tubes pre-coated with heparin (5000U / ml). Samples were kept on ice before being centrifuged (1,800 *g*, 20 minutes, 4^0C) with the subsequent plasma collected and stored at -20^{o}C for later quantification using commercially available ELISA kits as detailed in Table 2-13 below, following the manufacturers' instructions.

Following terminal blood collection tissues including pancreata and placentae were dissected and were either fixed in 4% formaldehyde solution (Sigma, UK) for histology or immediately snap frozen in liquid nitrogen prior to storage at -80^{o}C for RNA extraction.

Peptide	Supplier	Product No.
CRH	Cloud-Clone Corp, USA	CEA835Mu
UCN1	Cloud-Clone Corp, USA	CEA231Mu
UCN2	ELISAGenie, UK	MOFI00425
UCN3	Cloud-Clone Corp, USA	CED140Mu

Table 2-13 Supplier details for mouse CRH and Urocortins ELISA kits.

2.7 Histology

Histological techniques were employed to assess the consequence of blocking endogenous CRH ligands during pregnancy on β-cell proliferation. Excised pancreata from mice administered BrdU in the drinking water were immuno-probed using anti-BrdU and anti-insulin antibodies.

Immunohistochemistry (IHC) uses antibodies which recognise a specific part of the protein of interest (or antigen). The antibodies are detected with a reporter label, such as a fluorophore (fluorescent IHC), which allows the antibody-antigen interaction to be visualised by microscopy. Ultimately, information on the expression levels and localisation of proteins is enabled.

2.7.1 Tissue fixation and embedding

As mentioned previously, dissected pancreata were fixed in 4% formaldehyde solution (Sigma, UK) for 48 h. During the incubation in formaldehyde solution, the tissue is slowly penetrated by the fixing agent, cross-linking cellular proteins and thus allows preservation of tissue components. To enable the tissue to be infiltrated with molten paraffin wax, the tissue must first undergo dehydration by immersion in a series of ethanol solutions of increasing concentration. Therefore, the tissue was immediately transferred to 70% ethanol after fixation and then processed for paraffin wax embedding using an automated tissue processing machine (Leica TP1020, Leica Biosystems, UK). Each tissue specimen was placed in plastic cassettes before being rotated in a series of 2-hour time coursed solutions (for total processing time of 20 hours) as follows; 1. 70% ethanol, 2. 90% ethanol, 3. 100% ethanol, 4. 100% ethanol, 5. 100% ethanol, 6. 1:1 ethanol:xylene, 7. xylene, 8. xylene, 9. paraffin wax, 10. paraffin wax. Tissues were then transferred into individual metal moulds, layered with additional molten paraffin wax to form wax blocks and allowed to cool overnight.

2.7.2 Sectioning

A microtome (Leica RM2255, Leica Biosystems, UK) was used to cut paraffin blocks into 5 µm thick sections. A wax ribbon of approximately 2-3 consecutive sections were cut and transferred to the surface of ultrapure water in a 37ºC water bath to allow sections to flatten out. Sections were subsequently mounted on microscope slides (SuperFrost^{TM}, ThermoFisher Scientific, UK) and stored on a slide rack to dry overnight.

2.7.3 Immunohistochemistry

Fluorescent immunohistochemistry techniques were used to co-stain pancreas sections for insulin and BrdU. A total of 3 – 4 representative sections throughout the pancreas were stained per animal. Slides were briefly heated before sections were completely dewaxed by gentle agitation in xylene (2x 5mins). Sections were then rehydrated in a series of ethanol washes of decreasing concentration: 100% ethanol- 2mins, 95% ethanol- 2mins, 75% ethanol – 2mins. This was followed by a final wash in running tap water for 10 minutes to remove alcohol residue. Sections were then washed in Tris buffered saline (TBS -1x) (Table 2-14) and then each individual section outlined by a hydrophobic barrier using a wax pen. As BrdU detection requires denaturation of DNA, hydrochloric acid heat-induced and trypsin enzymatic antigen retrieval was applied. Slides

were therefore placed in a humidifying chamber and 2N hydrochloric acid pipetted onto each section and incubated for 20 minutes at 37ºC. Slides were again washed in TBS (1x) for 5 minutes followed by incubation (15 mins, 37ºC) with 0.05% trypsin. A further 3 washes (5 minutes each) in TBS were completed followed by the incubation of tissue sections with blocking buffer (Table 2-15) for 15 minutes at room temperature to minimise antibody non-specific interactions. Primary antibodies (mouse monoclonal anti-BrdU antibody, 1:100, Sigma, UK and guinea pig polyclonal anti-insulin antibody, 1:200, Dako, UK) diluted in blocking buffer were subsequently pipetted onto tissue sections and left to incubate in the humidifying chamber for 2 h at 37ºC.

Slides were then washed in TBS (1x) (3x 5mins) prior to the simultaneous incubation of sections with secondary antibodies (Alexa-Fluor 488, 1:50 and Alexa-Fluor 594, 1:50, Jackson, UK) for 1 h at room temperature ensuring sections were protected from light. Slide were then briefly washed in TBS (1x) and finally coverslips were placed on sections using fluorescent mountant and left to dry (protected from light) before imaging.

2.7.4 Imaging and morphometric analysis

Images were acquired using a Nikon Eclipse TE2000-U microscope and NIS elements software at 20x magnification. All islets on each section were analysed. ImageJ image analysis software was used to count the number of $BrdU^+$ β cells, total number of β cells, and cross-sectional area for each islet.

2.7.5 Buffer composition for immunohistochemistry

The summary tables below show the composition of the various buffers used in immunohistochemistry protocols.

Tris Buffered Saline (TBS) [10x]

Reagent	Amount (g/ 1l)
Trizma base (Supplier)	60.00
Sodium chloride	88.00

Table 2-14 Preparation of Tris Buffered Saline (TBS) [10x]. Reagents were dissolved in 500 ml of distilled water before being made up to a final volume of 1l with distilled water. The pH of the solution was adjusted to 7.6 with concentrated HCl and stored at room temperature.

Blocking buffer

Reagent	Amount
Bovine serum albumin (BSA) (Sigma, UK)	1% w/v
Goat serum (Sigma, UK)	10% w/v
TBS (1x) (Table 2-14)	-

Table 2-15 Composition of blocking buffer used in immunohistochemistry protocol.

2.8 Clinical study in pregnant women

Routine screening for gestational diabetes in pregnant women is generally conducted between 24-28 weeks' gestation using the one-step 75g- 2-hour oral glucose tolerance test (OGTT) (Consensus Panel, 2010). Similar to the glucose tolerance test described earlier for mice, venous blood samples can be taken from participants following a fasting period (baseline) and then at regular intervals for 2 hours following oral ingestion of a glucose solution. Measurement of blood glucose levels throughout the test allows for the monitoring of tolerance and response of islets to glucose as well as the diagnosis of gestational diabetes if certain criteria are met. Despite the test being time consuming, it is minimally invasive and has a high sensitivity (~92%) (Saranya et al., 2018). Simultaneously, additional blood can be drawn from participants for the measurement of various hormonal parameters throughout the test such as insulin or other hormones of interest. This can then allow for the study of possible changes in these hormone levels which may be associated with abnormalities in glucose tolerance during pregnancy.

Pregnant women undergoing routine oral glucose tolerance tests were invited to participate in the clinical study. The study was conducted in accordance with the Declaration of Helsinki (2013) and was approved by the London-Westminster Research Ethics Committee (13/LO/0539). A total of 91 women participated in the study and written informed consent was obtained from all participants.

2.8.1 Collection of human samples

Sample collection and preparation was conducted by a clinical research nurse at King's College Hospital. Pregnant women between 26- and 34-weeks' gestation underwent a standard oral glucose tolerance test (2-h -75 g) with additional blood sampling. Following an overnight fast (>9 hours), an intravenous cannula was inserted in an arm vein for blood sampling. Venous blood samples were taken before glucose consumption for the measurement of plasma glucose, serum insulin and any additional hormones (including CRH, UCN1, UCN2 and UCN3). The participant then drank 75 g glucose in 300 ml and rested for 2 h. Further blood samples were taken at 10, 60- and 120-minutes following glucose consumption for the measurement of plasma glucose and

serum insulin. Gestational diabetes mellitus (GDM) was diagnosed according to the IADPSG criteria as follows: fasting plasma glucose ≥ 5.1 mmol/l, 1-h plasma glucose ≥10.0 mmol/l or 2-h plasma glucose ≥8.5 mmol/l (Consensus Panel, 2010). One or more of these values from a 75 g OGTT must be met for diagnosis of GDM (Consensus Panel, 2010).

2.8.2 Preparation and assay of human samples

For plasma glucose measurement, venous blood samples (2 ml) were collected into BD Vacutainer Blood Collection Tubes (containing Fluoride EDTA). Samples were then centrifuged (855 *g*, 10 minutes, 4˚C) and plasma glucose was measured immediately using a YSI 2300 Stat Analyser (YSI Life Sciences, USA).

For serum insulin measurement, venous blood samples (5 ml) were collected into SST BD Vacutainer Blood Collection Tubes (containing Fluoride EDTA). Samples were allowed to stand for a minimum of 20 minutes at room temperature before centrifugation (855 *g*, 10 minutes, 4˚C). Serum was collected and stored at -80˚C until measurement of insulin levels using a commercially available insulin ELISA kit (10-1113-01, Mercodia, Sweden) according to the manufacturer's instructions.

2.8.3 Assay of human samples for CRH peptides using enzyme-linked immunosorbent assay (ELISA)

The enzyme-linked immunosorbent assay (ELISA) is a quick and sensitive immunological assay designed to detect and quantify a target antigen (e.g. protein) in a biological sample. The assay relies on the specificity of antigen -antibody interactions and requires the generation of a standard curve using known concentrations of antigen to determine the concentration of antigen in a sample. Essentially, the target antigen is immobilised to the surface of a pre-coated microtiter plate via an antibody specific to the antigen. The antigen-antibody complex is then recognised by a detection antibody which has been conjugated with an enzyme label. The most commonly used enzyme labels are horseradish peroxidase (HRP) and alkaline phosphatase (AP). Subsequent addition of an enzyme substrate (e.g. 3,3',5,5'-Tetramethylbenzidine (TMB) for HRP and p-Nitrophenyl Phosphate (PNPP) for AP) allows for its conversion to a detectable coloured product (known as a colorimetric assay) which is then detected using a microplate reader. ELISAs can be found in different formats depending on the method of antigen capture and detection. Two of the commonly used formats are sandwich and competitive ELISAs (Figure 2-7). In the competitive ELISA, the target antigen in the sample competes with an enzyme/biotin labelled antigen (competing molecule) for binding to a limited number of specific antibodies typically pre-coated on the microplate. After incubation, the unbound labelled antigen is washed off and addition of substrate solution permits a reaction, producing a coloured product. As the bound competing

antigen is responsible for generating the colour intensity, the signal output is inversely correlated with the concentration of antigen in the sample. The sandwich ELISA technique measures a target antigen between two layers of antibodies (capture and detection antibody) which bind to non-overlapping epitopes on the antigen. A secondary antibody (conjugated to an enzyme label) is added which recognises this sandwich complex and converts a substrate solution to a coloured product thus allowing detection. In this assay, the output signal is directly proportional to the concentration of antigen in the sample. ELISA kits for the measurement of mouse or human CRH, UCN1, UCN2 and UCN3 were either competitive or sandwich immunoassays.

For the measurement of plasma CRH, UCN1, UCN2 and UCN3 in pregnant women, venous blood samples (5 ml) were collected into BD Vacutainer Blood Collection Tubes (containing EDTA and Trasylol). Following centrifugation (855 *g*, 10 minutes, 4˚C), plasma was collected and stored at -80˚C. Plasma samples were subsequently assayed for CRH peptides using commercially available ELISA kits (detailed in Table 2-16) according to the manufacturer's instructions.

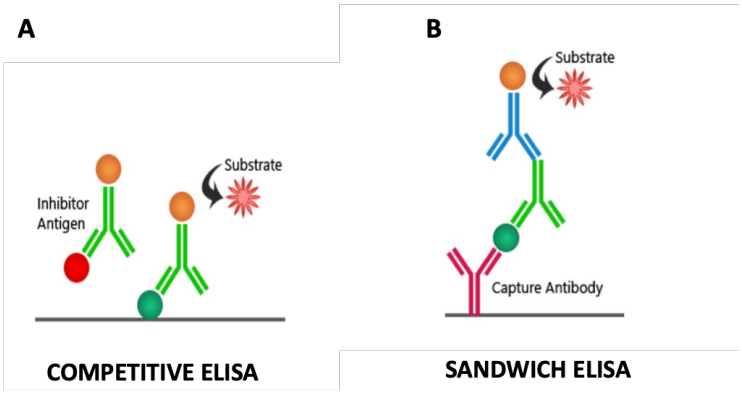

Figure 2-7 Schematic of the principles of competitive (A) and sandwich (B) ELISAs
Image modified from https://www.bosterbio.com/protocol-and-troubleshooting/elisa-principle

Peptide	Supplier	Product No.
CRH	Cloud-Clone Corp, USA	CEA835Hu
UCN1	Cloud-Clone Corp, USA	SEA231Hu
UCN2	Cloud-Clone Corp, USA	SEC585Hu
UCN3	Cloud-Clone Corp, USA	CED140Hu

Table 2-16 Supplier details for human CRH and Urocortins ELISA kits.

2.9 Statistical analysis

All statistical analyses were performed using GraphPad PRISM 8.0 software. For comparison between two-groups, two-tailed Students *t*-test was used. For *in vivo* glucose and insulin tolerance tests, two way repeated-measures analysis of variance (RM-ANOVA) was used, followed by Tukey's multiple comparison test to identify the significance between multiple groups. For analysis of correlations the Pearson product-moment correlation coefficient was calculated. All graphs were plotted using GraphPad PRISM 8.0 software. Any additional details regarding data presentation and analysis will be provided in the relevant results chapter/figure legend.

Chapter 3

Some data presented in this chapter has been published in the following journal;

Simpson, S.J.S., Smith, L.I.F., Jones, P.M., Bowe, J.E., 2020. UCN2: a new candidate influencing pancreatic β-cell adaptations in pregnancy. J. Endocrinology. 245, 247-257. https://doi.org/10.1530/JOE-19-0568

Chapter 3 Characterising the effects of the CRH system on pancreatic islet function *in vitro*

Chapter snapshot

A functional CRH receptor (CRHR) system expressing both CRHR1 and CRHR2 has been described in the endocrine pancreas.

A few studies have also revealed that corticotropin releasing hormone (CRH) and urocortins (UCNs) can directly augment islet hormone secretion from pancreatic islets although some of the literature is inconsistent.

Most studies have utilised male islets/mice to study the influence of CRH and CRH-related peptides on islet hormone secretion however little is known about the effects of these peptides on female islets.

Therefore, the aim of this chapter was to characterise the effects of CRHR activation on isolated male and female mouse islet function.

3.1 Introduction

The CRH system (including its structurally related peptides and cognate receptors) is best known for its important role in governing physiological responses to stress, as part of the hypothalamic-pituitary-adrenal (HPA) axis. Within the brain CRH is considered to be the principal stress transducer with the CRH-like peptides (UCN1, -2 and -3), integrating and fine-tuning responses which can either be complementary or distinct to that of CRH (Steckler and Holsboer, 1999). The CRH receptor (CRHR) system comprises two major types of cell surface GPCR; CRHR1 and CRHR2. These display differential distributions and expression densities throughout the CNS, particularly in the anterior pituitary (Steckler and Holsboer, 1999), as well as distinct binding specificities for the different members of the CRH family of ligands. CRH itself exhibits greater affinity for CRHR1 than it does CRHR2, with human/rat CRF (h/rCRF) reported to have a 4 – 20-fold greater affinity for CRHR1 than for CRHR2 (Hauger et al., 2006). However, UCN1 binds to both subtypes of receptor with similar affinity and with considerably greater affinity than CRH

(Hauger et al., 2006, 2003b; Takefuji and Murohara, 2019; J. Vaughan et al., 1995). Therefore, CRH and UCN1 are considered to be the endogenous ligands for CRHR1. Conversely, UCN2 and UCN3 are highly selective for CRHR2, though UCN2 displays a slightly higher affinity for this receptor (Hauger et al., 2006, 2003b). Functional studies using receptor overexpression in transfection systems have confirmed that activation of both CRH receptors is primarily coupled to Gαs and adenylyl cyclase signalling pathways. Therefore, in most tissues, stimulation of CRHR1 or CRHR2 by CRHR ligands, stimulates increases in intracellular cAMP levels to drive their physiological functions (Grammatopoulos, 2012; Markovic et al., 2011). CRH receptors are also believed to activate at least 4 different G-proteins with varying degrees of potency and may therefore modulate diverse signalling cascades in various target tissues. This appears to be a tissue- and receptor subtype effect although the factors/mechanisms determining CRHR- G-protein coupling are currently unknown (Grammatopoulos and Chrousos, 2002).

Although the CRH family is largely characterised as a neuroendocrine system, CRH receptor circuits independent to that of HPA axis signalling pathways have also been reported in peripheral tissues. Expression of CRHR1 predominates in the anterior pituitary and specific brain regions, however low mRNA expression levels have also been reported in the skin, gonadal tissue, adrenal glands, placenta, pancreatic islets and the gastrointestinal tract (Amisten et al., 2017; Boorse and Denver, 2006; Hauger et al., 2003b; Tsatsanis et al., 2007; Yang et al., 2010). CRHR2 is more widely expressed in the periphery with mRNA expression reported in various tissues including the spleen, thymus, gastrointestinal tract, pancreatic islets and an abundance in the heart, skin and skeletal muscle (Amisten et al., 2013; Baigent and Lowry, 2000; Coste et al., 2002; Lovenberg et al., 1995a; Slominski et al., 2004; Wypior et al., 2011). Differential peripheral expression of the CRHR splice variants also exists. Of the various CRHR isoforms that have been reported, CRHR1α (being the main fully functional CRHR1 isoform) and to a larger extent CRHR2β, are the predominant peripheral receptor isoforms in rodents. CRHR2α is the major splice variant found in the periphery in humans (Dautzenberg and Hauger, 2002). The wide tissue distribution of CRHR's suggests that the CRH family may have distinct biological roles in various peripheral tissues. Thus, a number of studies have begun to reveal the contribution of peripheral CRHR signalling in a wide spectrum of cardiovascular, metabolic, immune, gastrointestinal and reproductive responses, among others (Boorse and Denver, 2006; Yang et al., 2010; Ż mijewski and Slominski, 2010).

Several studies have reported components of the CRH system (both receptors and ligands) expressed throughout the gastrointestinal (GI) tract in both humans and rodents (Buckinx et al., 2011; Chatoo et al., 2018; Larauche et al., 2009). Generally, CRH receptors are expressed in proximity to their ligands in intestinal cells indicating that autocrine or paracrine CRHR signalling is involved in intestinal physiology via peripheral mechanisms. Activation of CRH receptors in

the GI tract have been shown to influence the integrity of the intestinal epithelial barrier and motor function, increasing ion secretion and mucosal permeability and stimulating colonic motility respectively (Larauche et al., 2009). However, CRH signalling pathways are more commonly implicated in a direct immunomodulatory role of gut inflammation (Kiank et al., 2010; Paschos et al., 2009; Rodiño-Janeiro et al., 2015) where activation of CRHRs results in the promotion of a proinflammatory response. Although the direct mechanisms are not completely understood, they may include mast cell activation via a CRHR-dependent mechanism as well as UCN2-induced NFkB signalling via CRHR2, resulting in an exaggerated release of proinflammatory chemokines (Bonaz and Bernstein, 2013; Kiank et al., 2010; Moss et al., 2007). As such, CRH and CRH-related peptides have been associated with the pathophysiology of irritable bowel disease (IBD) where intestinal inflammation is a main component of the condition (Gross and Pothoulakis, 2007).

An emerging field where local CRH systems are being extensively studied is in cardiac physiology. CRHR signalling in the heart has been associated with potent cardioprotective effects. In rodents, sheep and humans, stimulation of cardiac CRHRs *in vitro* and/or *in vivo* have shown to protect cardiac myocytes from hypoxia induced cell death, increase cardiac output and contractility as well as increasing heart rate (Davidson et al., 2009). The major direct effects on the heart however appear to be mediated by the urocortins instead of CRH as the peptide is not highly expressed locally in cardiomyocytes compared to urocortin peptides and CRHR2 (Davidson et al., 2009).

The pancreas is another organ where components of the CRH system have been reported (Li et al., 2003; Schmid et al., 2011). Initial evidence that CRH was synthesised in the pancreas was revealed by CRH-like immunoreactivity detected throughout the entire islet tissue (Petrusz et al., 1983). Subsequently, immunohistochemical staining of rat pancreatic tissue using CRHR antibodies revealed the presence of both type 1 and type 2 CRH receptors in the majority of islet cells. CRHR2 antibodies stained islet cells more weakly than did CRHR1 antibodies suggesting a greater abundance of type 1 receptors in the pancreas (Kanno et al., 1999).

Early reports attempting to elucidate the physiological effects of CRH on islet hormone secretion have been contradictory. Kanno et al demonstrated that in the presence of glucose (2.8 mM), application of CRH (2 nM) caused an increase in intracellular calcium in 40% of the single rat islet β-cells tested. Increasing the glucose concentration to 5.6 mM resulted in an increase in the percentage (50%) of β-cells exhibiting elevated $[Ca^{2+}]i$. The increase was not observed in Ca^{2+}-free extracellular solution, suggesting that CRH promoted the influx of Ca^{2+} into the cells, which would support a potential mechanism for CRH to stimulate insulin secretion (Kanno et al., 1999). However, earlier reports in various species and experimental situations suggested that CRH has

an inhibitory effect on islet β-cells. In separate studies Moltz and colleagues either reported an inhibitory action of CRH on insulin secretion in perfused rat pancreas (Moltz and Fawcett, 1985b) or no significant effects on insulin release *in vitro* from isolated rat islets (Moltz and Fawcett, 1985a). Intravenous injection of CRH (4.0 nmol/kg) into female mice resulted in time dependent effects on insulin secretion whereby plasma insulin levels were lowered at 2- and 6- minutes after CRH injection but elevated at 10 minutes after injection. The authors concluded however that CRH is of no great importance for the regulation of islet hormone secretion *in vivo* in the mouse (Karlsson and Ahrén, 1988). Studies in humans have also demonstrated no significant changes in plasma insulin following an intravenous bolus injection of CRH, although this was a very small study in 6 males only (Lytras et al., 1984).

Subsequently researchers have continued attempting to uncover the functional role of the CRH system within pancreatic islets. Accumulating evidence, consistent with observations from Kanno and colleagues' findings, have convincingly demonstrated that CRH can directly enhance insulin secretion from β-cell lines or pancreatic islets in addition to other positive influences on islet function. Confirming earlier discoveries, researchers have demonstrated mRNA and protein expression of CRHRs in rat, mouse and human islets with expression specifically shown in β-cell lines (Amisten et al., 2013; Huising et al., 2011, 2010; Schmid et al., 2011). As early as 2003, studies began to emerge showing that exogenous CRH and UCN3 (at relatively high concentrations-100 nM) could stimulate insulin as well as glucagon secretion from isolated rat islets (Li et al., 2003). It was suggested that secretion of these islet hormones is mediated primarily by CRHR2 as the stimulatory effects of CRH appeared to be less effective than the selective CRHR2 ligand, UCN3. Shortly afterwards, CRH-induced insulin secretion was shown in isolated mouse pancreatic islets although these authors failed to replicate the effects of UCN3 to stimulate insulin secretion (O'Carroll et al., 2008). Again, a high concentration of 50 – 100 nM CRH was required to stimulate insulin secretion and these stimulatory effects were proposed to be via CRHR1, as pre-incubation of islets with a selective CRHR2 antagonist (Astressin 2B) failed to block the observed induced insulin secretion. Additionally, the authors also showed that the stimulatory effects of CRH on insulin secretion were potentiated by arginine vasopressin (AVP) (a neurohypophysial antidiuretic hormone) analogous to a mechanism already recognised in pituitary corticotrophs whereby AVP potentiates the release of ACTH induced by CRH (Lee et al., 2015; O'Carroll et al., 2008).

Subsequent studies by Huising et al (discussed in more detail below) have further characterised the CRH-mediated effects on the endocrine pancreas (Huising et al., 2010). It was confirmed using a mouse β-cell line (MIN6) that CRHR1 mRNA expression levels were relatively abundant in islet β-cells compared to other islet cell types (Huising et al., 2010). Consistent with previous reports, activation of pancreatic CRHR1 with receptor-1 selective agonists (i.e. ovine CRF

(oCRF), rat/human CRF (r/h CRF) or Stressin I) in MIN6 cells or primary mouse and human islets, dose dependently promoted insulin secretion. This potentiation of insulin secretion only occurred in the presence of intermediate (11 mM) or high (16.8 mM) glucose concentrations however, unlike previous *in vitro* studies, much lower concentrations of CRH were required (i.e. 1 and 10 nM) (Huising et al., 2010). Importantly, it was shown that the augmentation of GSIS by CRH was primarily CRHR1-dependent as coadministration with the CRHR1-selective antagonist (Antalarmin) or the general CRHR antagonist (Astressin) completely inhibited the potentiation of insulin secretion by oCRF. The CRHR2-selective antagonist Astressin 2B only partially suppressed the actions of oCRF on insulin secretion (Huising et al., 2010) consistent with the effects of CRH on islet insulin secretion primarily acting through CRHR1, though the partial inhibition may suggest that CRHR2 may also play a role in this pathway. Additional *in vitro* studies showed that stimulation of MIN6 cells with oCRF dose dependently increased intracellular cAMP levels and caused phosphorylation of cAMP response element binding (CREB), thus enabling its nuclear translocation to initiate downstream transcriptional responses (Huising et al., 2010). The intracellular signalling pathways activated by CRHR in the β-cells are not restricted to cAMP signalling but additionally involve the activation of the mitogen-activated protein kinase (MAPK) signalling cascade. Consistent with MAPK activation, incubation of dissociated primary rat neonatal β-cells with CRH *in vitro* induced proliferation (as measured by the incorporation of 5-ethynl-2'-deoxyuridine (EdU)), suggesting a potential role of CRH in the augmentation of β-cell mass (Huising et al., 2010). Interestingly, the islet CRH response *in vitro* is reminiscent of the actions of incretins (i.e. GIP and GLP-1) on islets. This is not so surprising given the incretin receptors, GLP-1R and GIPR belong to the same subfamily of GPCRs that includes CRHR1 (i.e. Class B) (Hollenstein et al., 2014). Therefore, these hormones/peptides may exert similar effects via the activation of common downstream signalling cascades.

Finally, using *in vivo* approaches (such as CRHR1-null mice or adrenalectomized mice administered with CRHR1-selective peptide agonist Stressin 1) Huising et al concluded that pancreatic CRHR1 signalling *in vivo* has a positive influence on GSIS. Consistent with the *in vitro* data, administration of Stressin I to adrenalectomized mice had no effect on fasting insulin levels yet significantly increased plasma insulin levels following glucose challenge compared to vehicle-treated controls (Huising et al., 2010). Mice lacking CRHR1 exhibited significantly lower fasting- and glucose-induced plasma insulin concentrations compared to age- and gender matched wildtype animals. Unexpectedly, CRHR1 null mice displayed slightly improved glucose tolerance when compared with age-matched controls in a glucose tolerance test. However, it was suggested that this observation may be explained by the lack of a functional HPA axis and absence of pituitary CRHR1 preventing the normal glucocorticoid-mediated inhibition of insulin action (Huising et al., 2010). Taken together, these *in vivo* experiments are consistent with the *in vitro*

data presented in the study and support a role for CRH and pancreatic CRHR1 in modulating insulin secretion from β-cells (Huising et al., 2010).

Shortly after the publication of this study in 2010, further evidence supporting the beneficial effects of CRH on pancreatic islets was presented (Schmid et al., 2011). Functional testing in INS-1 cells and rat islets exposed to CRH (0.1 nM) for 24- and 48 h before glucose stimulation, revealed a significant potentiation of glucose stimulated insulin secretion (at 16.7 mM glucose). Treatment with CRH also promoted insulin synthesis, inducing a 63% and 40% increase in total insulin content in INS1 cells and rat islets respectively (Schmid et al., 2011). CRH had a significant and dose-dependent effect on proliferation of INS-1 cells in which CRH enhanced proliferation by ~40% after 24 h incubation as revealed by bromodeoxyuridine (BrdU) incorporation. These proliferative effects were partly abrogated by Astressin (non-selective CRHR antagonist), suggesting that the effects of CRH on proliferation may be mediated by CRHR1 (Schmid et al., 2011). Finally the authors showed decreased numbers of pancreatic islets in CRH-negative ($CRH^{-/-}$) mice compared with wildtype mice, further supporting effects of CRH on pancreatic endocrine cell turnover/viability (Schmid et al., 2011). These data indicate that the functional CRHR system within the endocrine pancreas can positively influence islet morphology and function however the physiological relevance of these effects is still unclear.

Selective CRHR agonists and antagonists have been extremely valuable in aiding characterisation of the CRH system in islet biology as has been demonstrated in the studies presented above. Structure-activity relationship studies have led to several potent and long-acting CRH analogues with selective binding to either one of the CRH receptors. In the last 40 years approximately 1,500 CRH analogues have been reported and at least 15 different compounds are available by commercial suppliers, many of which have been used successfully in animal models (Rivier and Rivier, 2014). Aside from, CRH, oCRF or r/h CRF, Stressin I is the most commonly used CRHR1 agonist, whilst commonly used selective CRHR2 agonists include commercially available synthetic preparations of UCN2 or UCN3 peptides. However, a number of novel modified peptide agonists selective for CRHR2 (e.g. PEGylated UCN2) are under development or patent application (https://patents.google.com/patent/US20100130424A1/en). As CRH has been implicated in the pathogenesis of a number of stress related diseases, CRHR antagonists have become an increasingly attractive therapeutic approach for treating these conditions. Alpha-helical CRF_{9-41} (α-helical CRF_{9-41}) was the first non-selective CRHR antagonist to be developed and subsequently selective CRHR1 antagonists including antalarmin or CP-154,526 are now available (Ẓmijewski and Slominski, 2010). Several CRHR2-selective antagonists have also been developed, including Astressin 2B and Antisauvagine-30 (Rivier and Rivier, 2014).

Commercially available CRH analogues (i.e. CRHR agonists and antagonists) were used throughout this project with the overarching aim of investigating the physiological role for the interaction between the endogenous CRH family and the pancreatic islets. However, given the limited and inconsistent literature, it was first necessary to characterise the direct effects of the CRH family peptides and the roles of each CRH receptor subtype on islet function.

3.2 Materials and Methods

3.2.1 Islet isolation

Islets were isolated from either male or female CD1 mice (Charles River Laboratories, Harlow, UK) via collagenase digestion of the pancreas, as described in section 2.2. Islets used for functional studies were maintained in culture (RPMI-1640 (Sigma, UK), supplemented with 1% (v/v) L-glutamine, 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin (100 U/ ml penicillin, 0.1 mg/ ml streptomycin) (Sigma, UK) in a humidified incubator at 37^{o}C (95% air, 5% CO_2) for a minimum of 24 h prior to experiments.

3.2.2 RNA extraction and real time qPCR

RNA was extracted from frozen male and female CD1 islets as well as pituitary samples using the RNEasy Mini Kit (Qiagen, UK) as described in section 2.4.1. Once RNA had been quantified and reverse transcribed to cDNA (see section 2.4.1.1 and section 2.4.2) a total of 30 ng cDNA per well was used for real time qPCR reactions (see section 2.4.3 for full method). Mouse genes analysed in isolated islets and pituitary included *Crhr1* and *Crhr2*. Mouse *Gapdh* was used as the housekeeping gene and all genes of interest were normalised to relative expression of this gene. See Table 2-11 for QuantiTect® primer catalogue numbers.

3.2.3 Static insulin secretion assay

Isolated male and female islets were used for functional *in vitro* static insulin secretion assays (described in section 2.3.1) to characterise the effects of CRH receptor (CRHR) agonists and antagonists on islet function. Following a 1-h preincubation in 2 mM Gey and Gey buffer (see Table 2.4), islets were then incubated in Gey and Gey buffer supplemented with glucose (2 mM or 20 mM) and CRHR agonists or antagonist (summarised in Table 2.2) for 1 h at 37^{o}C. 300 µl of supernatant was collected and stored at -20^{o}C for later insulin quantification by radioimmunoassay (section 2.3.4.1).

3.2.4 Dynamic (glucose-stimulated insulin secretion) perifusion

Dynamic perifusion experiments (as described in section 2.3.2) were conducted on isolated male and female islets to investigate the acute effects (50 nM) and chronic (48 h) exposure of CRH (1 nM) on insulin secretion. Further characterisation of CRHR signalling was investigated in female islets using selective CRHR agonists (as detailed in Table 2.2). 30-40 islets were handpicked and loaded into each channel of the perifusion system, with a total of 3-4 separate channels representing each respective treatment group. Following a pre-incubation period with 2 mM glucose Gey and Gey buffer (see Table 2.4) to achieve baseline insulin secretion, islets were then

exposed to a regime of Gey and Gey buffer (supplemented with either 2 mM or 20 mM glucose) with or without specific CRHR agonists at a flow rate of 0.5 ml/ min. Fractions containing insulin secreted were collected at 2 minute intervals into a 96-well block and stored at -20ºC until later insulin quantification by radioimmunoassay (section 2.3.4.1).

3.2.5 Insulin content

To investigate the effects of chronic CRH exposure or CRHR blockade on insulin synthesis, isolated female islets were incubated in islet RPMI culture medium (RPMI-1640 (Sigma, UK), supplemented with 1% (v/v) L-glutamine, 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin (100 U/ ml penicillin, 0.1 mg/ ml streptomycin)) supplemented with CRH (50 nM) or the non-selective CRHR antagonist, α-helical CRF_{9-41} (1 μM) for 48 h (as described in section 2.3.3). Samples were collected and lysed (10 islets/ treatment replicate) and stored at -20ºC until insulin quantification by radioimmunoassay (section 2.3.4.1).

3.2.6 Insulin radioimmunoassay

Insulin concentrations from static insulin secretion, dynamic perifusion and insulin content experiments were quantified by insulin radioimmunoassay as described in section 2.3.4.1. A standard curve (0.04 – 10 ng/ml) including reference tubes was prepared alongside experimental samples according to table 2.3. Static insulin secretion samples were diluted 1:5 and insulin content samples 1:2,000 in borate buffer (Table 2.5) to allow for the detection limits of the radioimmunoassay. Perifusion samples were assayed neat.

3.3 Results

3.3.1 Effect of CRH and CRHR agonists/antagonists on islet function – Static insulin secretion

Real time qPCR confirmed mRNA expression of *Crhr1* and *Crhr2* in both male and female islets (Figure 3-1A). *Crhr1* expression was higher than *Crhr2* expression in islets (~3-8-fold,), an expression pattern that is analogous to that in the pituitary, a classical target for CRH. Female islets displayed a 2-fold higher expression level of *Crhr1* compared to male islets however, a comparable expression level of *Crhr2* was shown for both male and female islets.

To characterise the effects of islet CRHR activation on islet function, static incubation assays were performed using selective CRHR agonists. In male and female islets exogenous CRH treatment consistently showed no significant effects on insulin secretion at basal levels (2 mM) of glucose (Figure 3-1B, C & D). In male islets, at stimulatory glucose concentrations (20 mM), CRH (50 nM) consistently potentiated glucose-stimulated insulin secretion (GSIS) by approximately 50% compared to control islets, however this effect was significant in some experiments (Figure 3-1C) and not in others (Figure 3-1B & E). No further potentiating effect was observed at ten times this dose of CRH (Figure 3-1B). To elucidate which CRHR type may be mediating these effects, both male and female islets were independently exposed to selective CRHR agonists, Stressin I (selective for CRHR1) and UCN2 (selective for CRHR2) (Figure 3-1C & D). In male islets a significant potentiation of GSIS was displayed with Stressin I (100 nM) and although UCN2 exposure resulted in a similar trend towards enhanced insulin secretion, this did not reach statistical significance (Figure 3-1C). A different insulin secretory profile was exhibited in female islets (Figure 3-1D). No potentiation of GSIS was observed with either CRH or selective CRHR1 activation however, selective CRHR2 activation by UCN2 significantly enhanced GSIS (Figure 3-1D).

To further decipher the involvement of CRHRs in the insulin secretory response in male islets, selective CRHR antagonists were also incubated with islets in combination with CRH (Figure 3-1E). Consistent with previous static insulin secretion experiments, a glucose-dependent potentiation of insulin secretion was observed with CRH (50 nM) treatment. Antalarmin hydrochloride (selective CRHR1 blockade) had no effects on GSIS in itself but reduced the CRH potentiation of GSIS by approximately 50%. However, the selective CRHR2 antagonist (Astressin 2B) appeared to display a glucose-dependent insulinotropic effect on islets when administered by itself and no alterations to insulin secretion were observed when Astressin 2B was co-incubated with CRH (Figure 3-1E).

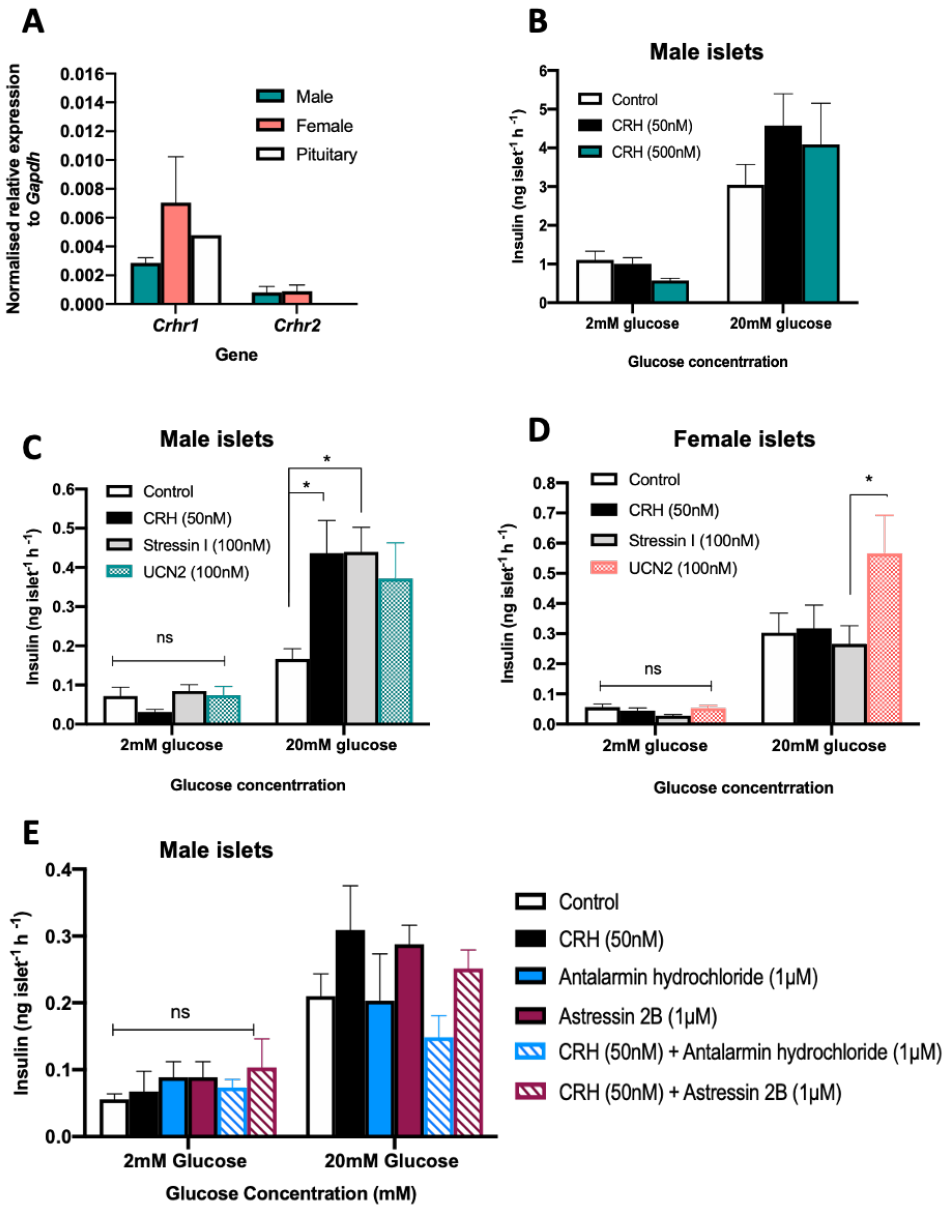

Figure 3-1 Pancreatic islet CRH receptor gene expression and effects of CRHR agonists/antagonists on islet function. mRNA expression levels of *Crhr1* and *Crhr2* in male (teal bar) and female (peach bar) mouse islets (A). Pituitary sample (white bar) was used as a positive control and mRNA expression levels were normalised relative to expression of housekeeping gene, *Gapdh* (mean ± SD, n= 2 biological replicates). Static insulin secretion CRH dose response in male islets (B) and in response to CRH receptor (CRHR) agonists in male (C) and female (D) islets are also shown. Effects of CRHR antagonists on static insulin secretion from isolated male islets (E). Data are presented as mean ± SEM, n = 5-10 replicates of islets pooled from 3-4 animals. *p <0.05, two-way ANOVA followed by Tukey's multiple comparison. ns=non-significant.

3.3.2 Effect of CRH on islet function and insulin synthesis – Dynamic insulin secretion

Although a few studies have implicated CRH in modulating insulin secretion *in vitro*, these have been mainly demonstrated using static incubation methods and in male islets. Dynamic perifusion studies were therefore employed to investigate the acute and chronic effects of CRH on male and female islet insulin secretion profiles and validate the observations seen in static incubations. For direct comparison of male and female islet responses to CRH, both were simultaneously exposed to CRH (50 nM) for a total of 20 minutes throughout the perifusion regime (Figure 3-2A). Islets from both sexes displayed the characteristic biphasic insulin secretion in response to 20 mM glucose. Male and female islets also exhibited a similar potentiation to GSIS following exposure to CRH which was reversible following removal of the agonist, although male islets appeared to have a slightly raised response compared to female islets (Figure 3-2A). Chronic (48 h) treatment of female islets to CRH (50 nM) or conversely with a non-selective CRHR antagonist (α-helical CRF_{9-41} -1 μM) did not augment insulin production as similar insulin content was measured between both treatment conditions and control islets (Figure 3-2B). Similarly, no differences in insulin content were observed in male islets following chronic exposure to CRH (data not shown).

Additionally, prior chronic exposure of male or female islets to CRH (1 nM) for 48 h had no effect on overall insulin release as demonstrated by the dynamic secretory profiles shown in Figure 3-2C & D. Both male and female islets responded similarly to basal and stimulatory glucose concentrations irrespective of chronic CRH treatment, with islets displaying a rapid spike in insulin secretion upon exposure to 20 mM glucose followed by a more prolonged second phase. Acute islet exposure to CRH (50 nM) throughout the perifusion potentiated GSIS by approximately 82% and 48% in male and female islets respectively although female control islets displayed a much more variable and delayed response throughout the perifusion (Figure 3-2C & D).

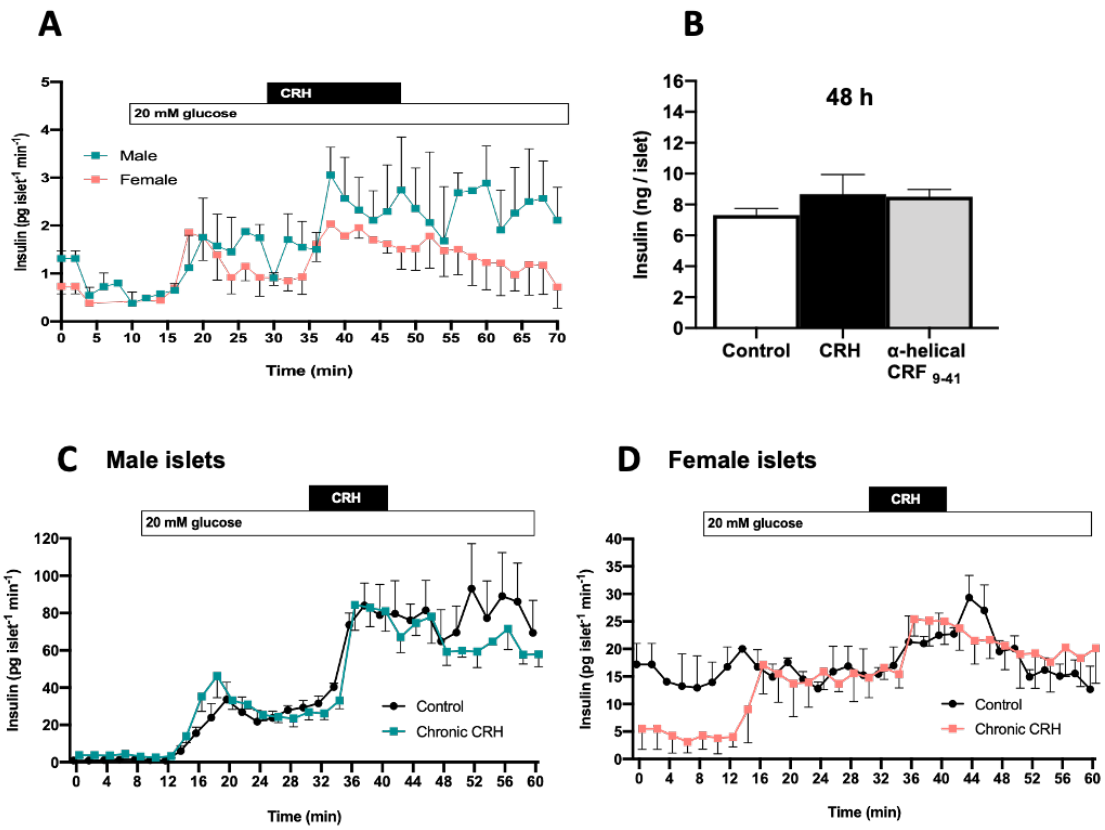

Figure 3-2 Dynamic insulin secretion profile of male and female islets following acute and chronic (48 h) exposure to CRH. Dynamic insulin secretion was investigated simultaneously in male (teal line) and female (peach line) islets acutely exposed to CRH (50 nM) (30- 50 min) (A). Measurement of insulin content following chronic incubation (48 h) with either CRH (50 nM) (black bar) or the non-selective CRHR antagonist, α-helical CRF_{9-41} (1 μM) (grey bar) (B). Male (C) or female (D) islets were incubated in the absence (control-RPMI) (black line) or presence of CRH (1 nM) (chronic CRH) for 48 h prior to dynamic perifusion experiments, where islets were also acutely exposed to G&G buffer supplemented with CRH (50 nM) between 30- and 40- min. Data are presented as mean ± SEM, n = 3-4 channels/treatment group containing 40 islets pooled from 3-4 animals for perifusion data. Insulin content; n= 5 replicates of pooled islets from 1-2 animals. Data analysed using one-way or two-way ANOVA followed by Tukey's multiple comparison.

3.3.3 Activation of female islet CRH receptors *in vitro*: Dynamic insulin secretion

Previous reports have suggested a role for CRHR2-specific ligands in modulating islet hormone secretion which was also suggested by our earlier static insulin secretion studies. To elucidate the pharmacodynamics of CRHR activation and subsequent insulin secretion, female islets were exposed to CRHR-specific agonists in dynamic perifusion experiments. Increasing the concentration from basal (2 mM) to stimulatory (20 mM) glucose concentrations initiated the expected insulin secretory response from islets as shown in Figure 3-3. The subsequent addition of 50 nM CRH in the presence of 20 mM glucose rapidly potentiated insulin secretion which was reversed following removal of the agonist (Figure 3-3A). Similarly, islet exposure to 100 nM of either Stressin I or UCN2 (CRHR1- and CRHR2-specific agonists, respectively) also enhanced GSIS (Figure 3-3C & E). The potentiation of GSIS (30 – 50 min) in comparison to control islets was further quantified using area under the curve analysis which confirmed the significant increase in insulin secretion in response to glucose induced by all CRHR agonists tested (Figure 3-3B, D & F).

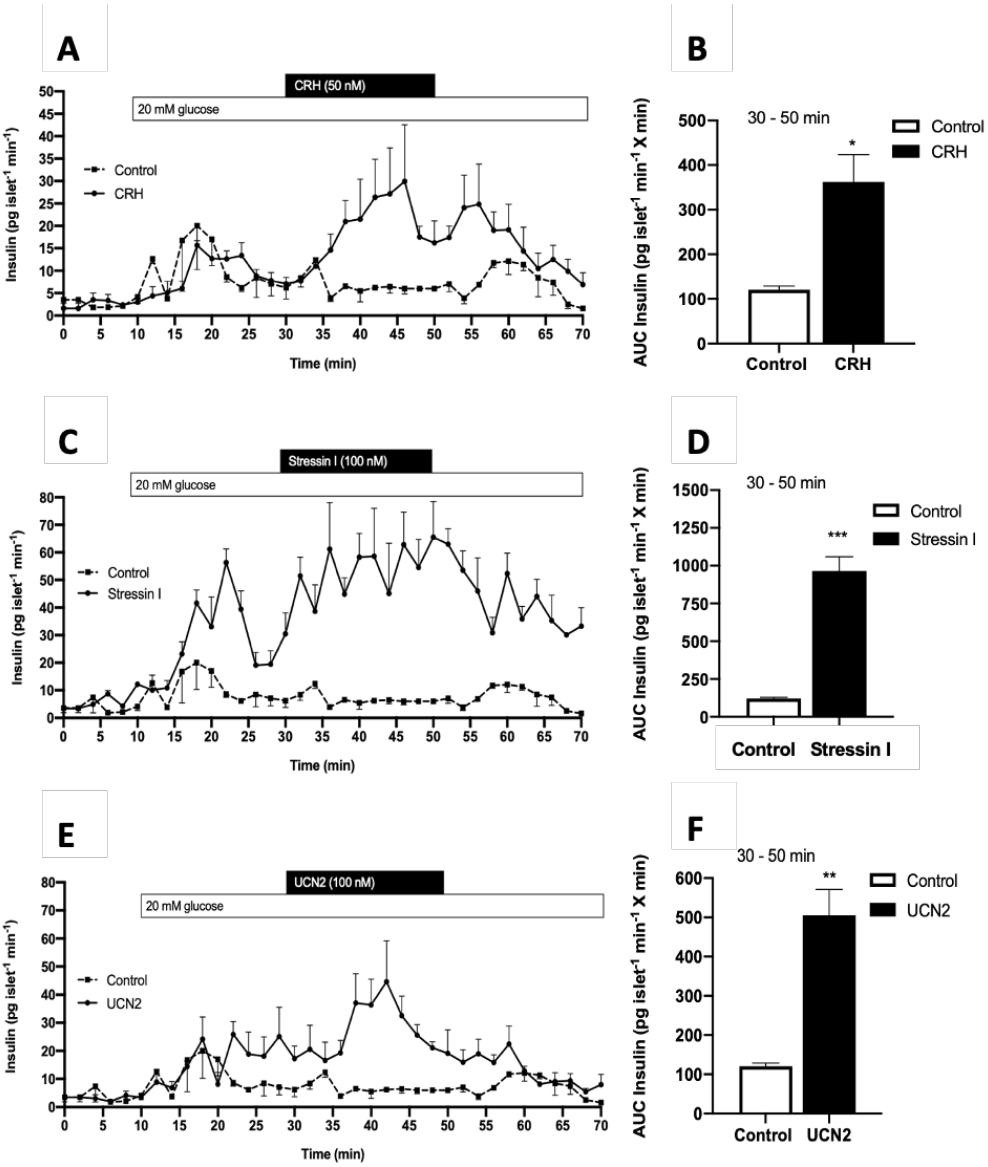

Figure 3-3 Effects of exogenous CRHR agonists on female islet insulin secretion. Dynamic insulin secretion from isolated, perifused female mouse islets was assessed following exposure to CRH (50 nM) (A), CRHR1-selective agonist, Stressin I (100 nM) (C) and CRHR2-selective agonist, UCN2 (100 nM) (E). Islets were exposed to physiological buffer (Gey&Gey) containing 20 mM glucose only or supplemented with agonists between 30- and 50-min. Representative AUC for glucose stimulated insulin secretion for control islets and islets exposed to each agonist are shown (B, D & F). Data are presented as mean ±SEM, n = 3-4 channels/treatment group containing 30-40 islets pooled from 3-4 animals. AUC 20 mM glucose -/+ agonist (control vs agonist) *p <0.05, **p < 0.01, ***p < 0.001, two tailed t-test.

3.4 Discussion

Since its initial characterisation within the brain, local CRH/urocortin networks within peripheral tissues have also been identified, alluding to the existence of supplementary roles for CRH ligands and receptor signalling independent from HPA circuits (Boorse and Denver, 2006; Ilias and Mastorakos, 2003; Kuperman and Chen, 2008). One such organ where components of the CRH system have been reported is the endocrine pancreas (Huising et al., 2010; Kanno et al., 1999; Li et al., 2003; Petrusz et al., 1983). A number of studies have reported mRNA or protein expression of CRHR1 and/or CRHR2 in β-cell lines (Huising et al., 2010; Kanno et al., 1999; Schmid et al., 2011), rodent islets (Huising et al., 2011; Schmid et al., 2011) and human islets (Amisten et al., 2013; Huising et al., 2011) consistent with the expression profile we report here in the current study. Analogous to the pituitary, CRHR1 is the dominant receptor subtype in both male and female islets suggesting that islet cells have an innate capacity to recognise and respond to local or circulating CRH ligands. Whilst the results appear to show higher CRHR1 expression in female islets in comparison to male islets, this expression profile isn't supported by recent RNA sequencing studies in mouse pancreatic β-cells (Stancill et al., 2018). Our experiments aimed to confirm that both CRHR1 and CRHR2 are expressed in both male and female islets but only involved two biological replicates, with female islets displaying high variability between samples. Nevertheless, the results support the key point that both receptors are expressed in the islets of both sexes and the secondary point that CRHR1 expression seems to be higher than CRHR2 which has previously been demonstrated in MIN6 cells and mouse islets (Huising et al., 2011).

Earlier investigations into the functional effects of CRHR activation to influence islet hormone secretion demonstrated either no significant effect or a dose-dependent inhibition of insulin release (Moltz and Fawcett, 1985a, 1985b). The more recent reports however have challenged these earlier reports. Contrary to the initial studies, CRH and CRHR agonists have consistently been shown to enhance insulin secretion from either β-cell lines and/or primary islets (Huising et al., 2010; Li et al., 2003; O'Carroll et al., 2008; Schmid et al., 2011). Accordingly, our *in vitro* static insulin secretion studies supported a functional role for CRHR activation to potentiate GSIS although there were some inconsistencies between independent experiments. Additionally, there were noticeable differences in the absolute insulin secretory values between the male islet static assays and CRH appeared to have a variable effect size on GSIS in independent experiments (ranging from a 1.5 – 2.3-fold increase). Although within each assay islets were consistently size-matched, intrinsic variability exists between different islet preparations which may also be influenced by the condition of the islets in the pre-culture period and the handling of islets throughout the assay. This may have contributed to the variable secretion profiles observed. Furthermore, CRHR1 expression has also been reported in other islet cell lines including α- and δ- cells (albeit not as abundant as the expression of the receptor in β-cells) (Huising et al., 2010).

Although the direct effects of CRH on these cells are largely unknown, it is possible that other intra-islet hormone secretion may influence the overall effect of CRH on GSIS. Nonetheless, in keeping with the literature, male islets demonstrated a consistent trend to enhance insulin release in a glucose-dependent manner with no changes to basal hormone secretion. Augmentation of insulin release, particularly by β-cell GPCR signalling is commonly exhibited at stimulatory glucose concentrations (and not at low glucose), thereby amplifying the insulinotropic effects of glucose itself. Islet CRHR activation mirrors the response of other biological hormones (e.g. GLP-1) to potentiate β-cell insulin secretion and this suggests that the physiological function of CRH ligands is to module the extent of the insulin secretory response to elevated glucose concentrations rather than to initiate secretion.

Our CRHR agonist studies suggest that either CRHR1 or CRHR2 activation could mediate the observable effects we saw on islet function. However, assays utilising CRHR antagonists indicated that the response to CRH itself may be CRHR1-dependent as selective blockade of CRHR1 inhibited insulin release in response to CRH. This finding also corresponds to the findings by Huising and colleagues (Huising et al., 2010) who also suggested the augmentation to GSIS by CRH was primarily CRHR1-dependent. However, the results from this assay are difficult to interpret as the selective CRHR2 antagonist, Astressin 2B, unexpectedly appeared to independently potentiate GSIS. Recently, it has been reported that Astressin 2B can act as a weak partial agonist at CRHR2 at concentrations as low at 10 nM and may explain these observations (Tasma et al., 2020). However, this result does not necessarily mean CRHR2 is not involved in stimulating insulin release but just suggests that CRH preferentially signals via CRHR1 to increase insulin secretion and perhaps CRHR2 may have a similar role but in response to other ligands.

Static insulin secretion experiments using female islets were much more inconsistent with findings in male islets, though it is not surprising that disparate responses were observed between the sexes. Much of the previous characterisation of CRH ligand influences on islet function has been conducted in male islets. However, at least one study in the literature has reported sexually dimorphic metabolic responses of mice *in vivo* mediated by CRHR2 (i.e. regulation of blood glucose levels, fat mass gain/redistribution and insulin resistance) (Paruthiyil et al., 2018), suggesting the possibility that local islet CRHR signalling may also exhibit distinct responses between the sexes. Whilst CRH or CRHR1 agonists had little effect on female islets in static incubations, activation of CRHR2 with UCN2 appeared to potentiate GSIS. Nevertheless, the discrepancies in these *in vitro* studies could also be a consequence of the static incubation technique itself. Although static incubations lend themselves to allowing a wide range of experimental conditions/treatment groups, they are limited by the inability to distinguish the multiphasic aspects of insulin secretion. Additionally, during the 1 h incubation, secretory

responsiveness to various agents may be influenced by the accumulation of other islet hormones. Therefore, it is possible that subtle increases in insulin secretion could be masked by local effects of inhibitory molecules such as somatostatin on insulin release. Given that islet CRHR2 activation has also been shown to stimulate glucagon and somatostatin release (Li et al., 2003; van der Meulen et al., 2015), these effects might interfere with the net action of CRH on GSIS and produce inconsistent results. Due to the inconsistent results achieved with static incubation experiments, dynamic perifusion studies were employed as a more sensitive method to validate the observations seen in static incubations.

Direct comparison of dynamic insulin secretion from both male and female islets confirmed the potentiating effect of CRH on GSIS which was reversible following removal of the agonist and is consistent with studies using mouse or human islets in static incubations (Huising et al., 2010; O'Carroll et al., 2008). As enhanced insulin secretory responses have been associated with increased insulin synthesis (Schmid et al., 2011), it was expected that chronic CRH treatment might increase islet insulin content and further amplify the insulin secretory response. However, no augmentation to islet insulin content was measured with either CRH or α-helical CRF_{9-41} (CRHR antagonist) treatment. Although Schmid and colleagues reported the increase in total insulin content (cellular insulin content + insulin release) in INS-1 cells after a shorter incubation (24 h) with a much lower concentration of CRH (an effect that was also reported to be reproduced in primary rat islet cells) our observations suggest that longer incubations with CRH does not appear to augment insulin production. Additionally, chronic treatment of male and female islets with CRH had no effect on the dynamic insulin secretory profile with both sexes exhibiting a similar potentiation of GSIS following acute exposure to CRH. However, prior chronic CRH treatment for 48 h did not modulate this potentiation of insulin release. Despite the female islets appearing to reveal no superior effects on potentiated insulin release with chronic CRH treatment, this statement may be limited due the control islets included in the assay hypersecreting insulin in the unstimulated (basal) state and not displaying the expected response to 20 mM glucose. Therefore, comparisons to the control treatment group may not be reliable and this experiment would need to be repeated to confirm this. It is possible that islets were "leaking" or hypersecreting insulin possibly due to disturbances to the integrity of the β-cell membrane which could be caused due to air bubbles arising in the perifusion system or the islets being stressed (Alcazar and Buchwald, 2019). Nevertheless, the evidence in male islets most likely suggests that prior chronic CRH treatment does not alter the insulin secretory profile of islets.

The fundamental physiological relevance of these functional effects within islets is still unclear. It is probable that the receptor isoforms do modulate distinct roles but without a full understanding of the physiological significance of islet CRHR signalling, it is difficult to make any assumptions. As presented in the general introduction (Chapter 1), there is some evidence that placentally

derived CRH and urocortins are involved in various biological functions associated with pregnancy. Although male and female islets demonstrate the capacity to enhance insulin secretion following CRHR activation in a glucose dependent manner, the observations from static and perifusion studies appeared to indicate possible differences in the mechanisms of this response between the sexes. From the initial experiments in the current study, it would seem as though male islets are more effective at potentiating GSIS via CRHR1 and CRH whereas in female islets CRHR2 and selective agonists against this receptor may be more involved. However, as discussed above, the static incubation results were inconsistent and not necessarily reliable. Before investigating a potential physiological role for the CRH family in pregnant islet signalling it was first necessary to clarify the effects of CRHR1 and CRHR2 activation in female islets specifically. Dynamic measurements of insulin secretion from isolated female islets reliably demonstrated that activation of either CRHR1 or CRHR2 significantly potentiates GSIS. Notably, each CRHR selective ligand had relatively large effects, increasing the amount of insulin secreted by 1.5 -3-fold more than the peak response of glucose alone which is comparable to that reported by other insulin secretagogues such as GLP-1 or Exendin-4 which have been reported to stimulate insulin secretion 3-4-fold more than glucose alone in isolated mouse islets (Peyot et al., 2009). The effects of CRH ligands to sustain the enhancement to insulin secretion are even more dramatic throughout the second phase of glucose induced insulin secretion, exhibiting a 5 – 10-fold increase in insulin secreted in comparison to controls. In comparison to the static insulin secretion studies, these effects are much more pronounced however, it is important to bear in mind that often higher doses of agonists are used during *in vitro* studies which may result in large effects but does not necessarily guarantee that the same magnitude of effect will be replicated in a physiological *in vivo* setting. Though it would seem a redundant mechanism to have both islet CRHRs mediate the same effects on β-cell insulin secretion, in principle activation of either receptor could play a physiological role in pregnancy. The fact that islets have the machinery to respond to CRH peptide ligands coupled with the evidence presented in this chapter and within the literature that islet CRHR activation can module insulin release in a favourable way, supports the potential involvement for members of the CRH family in pregnancy-induced islet adaptions. Therefore, the next chapter will investigate whether there is a physiological role for the CRH family during pregnancy.

Chapter 4

Some data presented in this chapter has been published in the following journal;

Simpson, S.J.S., Smith, L.I.F., Jones, P.M., Bowe, J.E., 2020. UCN2: a new candidate influencing pancreatic β-cell adaptations in pregnancy. J. Endocrinology. 245, 247-257. https://doi.org/10.1530/JOE-19-0568

Chapter 4 Investigating the physiological role for the CRH family and CRHR signalling during pregnancy

Chapter snapshot

Observations from the previous chapter (i.e. expression of islet CRHRs and the insulinotropic effects of CRHR activation) support a role for islet CRHRs regulating islet function although the physiological purpose of this is unclear.

Recently, mRNA expression levels of CRH ligands and CRHRs have been shown to be altered during pregnancy in the placenta and islets respectively, suggesting pregnancy may be a physiological state where the CRH system is involved in maternal islet adaptations.

Although some conventional mouse models of CRHR inactivation/deletion display metabolic phenotypes, the consequence of blocking endogenous CRHR ligands during pregnancy on glucose homeostasis has not yet been explored.

Therefore, the aim of this chapter was to investigate whether there is a physiological role for the CRH system during pregnancy by pharmacologically blocking endogenous CRH/UCN-peptide ligands during gestation and assessing the physiological consequence on maternal β-cell adaptations and glucose homeostasis.

4.1 Introduction

Murine pregnancy involves a series of critical processes which occur over a brief gestational period of 3 weeks compared to the 9-month gestational length in humans. Successful blastocyst implantation and placentogenesis, on gestational day 4.5 and 7-12 respectively, are shortly followed by parturition typically between day 18–21 (Ander et al., 2019; Blum et al., 2017). Tightly regulated morphological and functional alterations to maternal pancreatic β-cells are also sequenced into this gestational timeline (previously described in more detail in section 1.5.3). β-

cell mass expansion and hyperfunction are important compensatory adaptations to alterations in maternal insulin demand during pregnancy and have been well documented in rodents (Baeyens et al., 2016; Rieck and Kaestner, 2010; Sorenson and Brelje, 1997). Cellular processes involved in β-cell adaptations in rodent models include hypertrophic expansion, proliferation and enhanced glucose-stimulated insulin secretion (Ernst et al., 2011). Particularly in rodent models, maternal glucose clearance and *in vitro* morphometric analysis are often practical indicators of these metabolic adaptations which can also be used to investigate if disturbances in the β-cell adaptive response have occurred, as may be the case in gestational diabetes. In humans, the primary indicator of disturbances to glucose homeostasis is measured by oral glucose tolerance testing during the second trimester of pregnancy. In rodent models changes in maternal islet growth and function occur in a narrow gestational window with enhancement in islet function occurring on days 11-19 and maximal β-cell proliferation between gestational days 13-15 (Ernst et al., 2011; Rieck and Kaestner, 2010). As discussed in the general introduction (chapter 1), the β-cell adaptations in human pregnancy are not comprehensively understood due to the limited availability of human autopsy pancreas samples. As such, mapping out the β-cell adaptive events throughout human gestation is still an ongoing aim for researchers.

Placentally released hormones play important roles during many gestational events, including those related to islet adaptations to pregnancy. Among the molecular signals known to contribute to gestational β-cell adaptations, the lactogens are the most well studied. Initial observations by Parsons et al were influential in revealing the changes in islet cell proliferation and insulin secretion during gestation coincided with the increase in placental lactogen (PL) (Parsons et al., 1992). However, it is important to note that these correlations were mapped out using rodent gestational timescales and rodent lactogenic secretion profiles which do not entirely parallel changes in hormone levels during pregnancy in humans. In rodents, PL begins increasing mid gestation (~day 10) with a rapid decline around day 14, before surging to peak levels in the latter half of gestation (from day 15-18). Prolactin (PRL) increases exponentially from day 18 of pregnancy (Nadal et al., 2009; Soares, 2004). In humans, human placental lactogen (hPL) and PRL simultaneously begin to rise from week 12 of gestation reaching peak levels in the third trimester (~30 weeks) (Freemark, 2006; Nadal et al., 2009). Peak up-regulatory changes in the β-cell, at least in rodents, occur during mid-gestation (Rieck and Kaestner, 2010). In human pregnancy there is also evidence to suggest adaptive β-cell insulin secretory responses in early pregnancy (12-14 weeks) (Powe et al., 2019). While the onset of PL secretion in rodents and both PL and PRL coincide with detectable changes in β-cell proliferation and insulin secretion, the subtle differences in hormonal changes between species may suggest possibly some differences in their adaptive pathways (Moyce and Dolinsky, 2018; Nadal et al., 2009). Nevertheless, the serum lactogenic hormone levels during pregnancy point to their key role in the adaptation of the islets to pregnancy.

Pituitary-derived PRL and placentally produced PL, both signal via the prolactin receptor (PRLR) and have been shown to orchestrate key components of metabolic adaptations to pregnancy in rodents both *in vitro* and *in vivo* (Sorenson and Brelje, 2009). A substantial amount of evidence in support of the PRLR-dependent metabolic adaptations to pregnancy has derived from either β-cell specific overexpression of PLs or conversely, *in vivo* models of PRLR-deficiency (Freemark et al., 2002; Vasavada et al., 2000). Initial studies performed by Huang and colleagues, using a whole body heterozygous PRLR-knockout mouse model, constituted the first direct *in vivo* validation that lactogen signalling was required for normal functional β-cell mass expansion and maternal glucose tolerance during pregnancy. Pregnant $PRLR^{+/-}$ mice displayed impaired glucose clearance, decreased GSIS, as well as defects in β-cell mass expansion compared to wildtype mice (Huang et al., 2009). Further studies to delineate whether these observations were β-cell specific effects came from Banerjee and colleagues, who generated a mouse model with targeted PRLR inactivation specifically in β-cells. Their findings were significant in validating a critical role for β-cell PRLR signalling in rodent gestational β-cell adaptations and the authors were able to correlate the metabolic phenotype in these mice with a phenotype consistent with gestational diabetes (Banerjee et al., 2016b). Hence, the common theory that impairment/dysregulation to the signals mediating gestational β-cell responses are important in the pathophysiology of gestational diabetes.

Investigations into other candidate hormones involved in β-cell adaptive mechanisms have identified other molecules capable of inducing changes in β-cell physiology that mimic or complement those adaptations characterised in pregnancy. More recently, a classical neuroendocrine hormone, kisspeptin, has emerged as a placental derived hormone involved in mediating β-cell adaptive responses to pregnancy. The kisspeptin receptor, GPR54, is highly expressed in pancreatic islets and during pregnancy maternal plasma levels of kisspeptin rise exponentially from as early as the first trimester until term (Dhillo et al., 2006; Hauge-Evans et al., 2006; Horikoshi et al., 2003a; Kotani et al., 2001; Ohtaki et al., 2001). As with lactogenic hormones, kisspeptin has also been shown to positively influence β-cell function *in vitro* and *in vivo,* stimulating insulin release (Bowe et al., 2012, 2009; Hauge-Evans et al., 2006). Further support for placental kisspeptin in modulating the islet adaptations to pregnancy has been recently demonstrated by Bowe et al (2019). Studies by these authors revealed that pharmacological blockade of endogenous kisspeptin in pregnant mice or β-cell-specific GPR54 knock down during pregnancy resulted in glucose intolerance which was associated with a reduced insulin response to glucose and reductions to β-cell proliferation (Bowe et al., 2019). Together, these data suggest a role for kisspeptin in maintaining maternal glucose homeostasis during pregnancy and provides compelling evidence for involvement of additional signals other than the lactogens in islet adaptive responses to pregnancy.

Analysis of the transcriptional response of the islet to pregnancy has played an integral role in identifying potential signals which may be implicated in endocrine communication between the placenta and the islets to drive the adaptive response. Nearly 2,000 genes are differentially expressed in the pancreatic islet throughout gestation (Rieck et al., 2009). Alterations in gene expression patterns in pregnant pancreatic islets combined with data of known ligands expressed by the placenta has provided candidate genes that may be involved in placental–islet communication. It is reasonable to assume that signals involved might be expressed at high levels in the placenta and that altered expression of the relevant receptors would be observed in the islets when β-cell mass is actively expanding or when islet functional capacity is approaching peak levels. Levels of both the placental ligand and the islet receptors may also be downregulated towards the end of term. However, these associations can be difficult to tease out when such a high frequency of transcriptional alterations occur in the pregnant islet.

A recent study attempting to characterise the placental-islet crosstalk to identify novel placental ligands/islet GPCRs involved in pancreatic islet responses to pregnancy, was reported by Drynda and colleagues (2018). Unlike GPCR signalling, the PRLR is a type 1 cytokine receptor that initiates signalling cascades primarily via janus kinase (JAK)-signal transducer and activator of transcription (STAT) signalling pathways (Baeyens et al., 2016; Gorvin, 2015). However, GPCRs represent the largest class of cell surface receptor with mouse and human islets expressing 279 and 293 different GPCRs respectively, offering multiple opportunities to modulate islet hormone secretion (Amisten et al., 2017, 2013; Simpson et al., 2018). Therefore, screening placental ligands against islet GPCR libraries may offer a greater pool of potential novel signals that may be involved in adaptive responses of islets to pregnancy. As such, Drynda et al used real time qPCR arrays to measure mRNA expression levels of 342 GPCRs in islets from non-pregnant mice, and in islets isolated from mice on gestational days 12 and 18. Additionally, expression of 126 islet GPCR ligands in mouse placenta at gestational days 12 and 18 were also measured. Subsequently, an atlas of potential interactions between the placenta and β-cells was generated based on the expression of some islet GPCRs and placental ligand mRNAs that displayed altered expression by gestational day 18. Interestingly, one network that was identified was ligands and receptors belonging to the CRH family. Expression of islet CRHR1 and placental CRH, UCN2 and UCN3 were upregulated on gestational day 12 compared to gestational day 18 (Drynda et al., 2018), suggesting the CRH system may be involved in the endocrine communication between the placenta and islets. In support of this hypothesis are the results from the *in vitro* functional studies presented in chapter 3 of this thesis, demonstrating the ability of CRHR signalling to positively influence β-cell function. Collectively, these data support a potential role for components of the CRH system in the adaptive maternal response of the β-cell during gestation.

The CRH system is known to play a regulatory role in glucose homeostasis via both central and peripheral mechanisms (Kuperman and Chen, 2008). Genetically engineered mice targeting specific components of the CRH system have extended our knowledge on the biological stress, behavioural, inflammatory and metabolic responses mediated through both type 1 and type 2 CRH receptors. Unsurprisingly, many mutant mouse models focus on CRH peptide signalling within the CNS and the neurophysiological consequences of CRHR deficiencies. However, a few transgenic models have also exhibited intriguing metabolic phenotypes. Conventional CRHR1 knockout (CRHR1-KO) and CRHR2-KO mouse models both exist however, the offspring from CRHR1-KO homozygous matings leads to neonatal mortality (with 48 h of birth) as a result of inadequate lung maturation (Smith et al., 1998). The metabolic profile of CRHR1-KO mice generated from heterozygous mating pairs has been characterised by Sakamoto and colleagues. Mice lacking CRHR1 displayed a similar glucose profile to that of WT littermates in response to a glucose tolerance test however, plasma insulin levels were significantly lower in the knockout mice. Additionally, pancreatic islet hypoplasia and defective glucose stimulated insulin secretion (in isolated KO islets) was exhibited in CRHR1-KO mice, suggesting a role for CRHR1 in maintenance of β-cell mass and β-cell insulin secretory responses (Sakamoto et al., 2013). Studies conducted in CRHR2-deficient mice have revealed conflicting data. Knock out male mice are reported to have impaired glucose clearance, whilst others have reported improved glucose clearance and insulin sensitivity in mutant mice (Bale et al., 2003; Paruthiyil et al., 2018). These genetic models represent a supraphysiological event and it is often difficult to decipher the relative contribution of centrally versus peripherally mediated effects in driving the phenotypes. The generation of tissue-specific knockout models would be valuable in elucidating the roles of CRHR1 and CRHR2 in individual tissues but these are limited and represent an expensive, labour intensive and time-consuming approach. Pharmacological receptor antagonism provides a simpler alternative approach to studying endogenous receptor signalling *in vivo*. Several synthetic CRHR antagonists have been developed and used *in vivo* (injected intracerebroventricularly, intravenously or infused via osmotic minipump) and have contributed to our understanding of CRHR signalling as well as offering potential therapeutic strategies for various pathologies associated with the CRH family (Rivier and Rivier, 2014). In metabolic studies specifically, CRHR antagonists have been commonly used to validate the effects of local pancreatic CRH ligands and/or CRHRs on islet hormone secretion as well as in studies investigating the general consequences of either central or peripheral CRHR antagonism on overall metabolic regulation. Some of these studies have shown results consistent with selective CRHR-KO mouse models. For example, Li et al demonstrated that blocking endogenous CRHR2 signalling *in vivo* with Astressin 2B (injected intravenously into male rats 20 mins before glucose bolus) attenuated GSIS with plasma glucose levels mirroring these effects (Li et al., 2007). This corresponds to the impairment in glucose tolerance displayed by the CRHR2 knockout mice described by Paruthiyil et al (2018) although the phenotype in the knockout mice was only observed under HFD

conditions (Paruthiyil et al., 2018). Studies by Chen et al contradict the idea that inhibiting endogenous CRHR2 signalling (by CRHR2 KO or pharmacological antagonism) has detrimental effects on glucose clearance as administration of the CRHR2-specific antagonist Astressin 2B (to mice 20 mins prior to GTT) significantly improved glucose tolerance compared to WT mice administered saline (A. Chen et al., 2006), Furthermore, CRHR1-specific antagonists (i.e. Antalarmin) administered chronically to rats (i.p. for 11 days) did not induce any changes in metabolic status with mean morning blood glucose levels comparable between vehicle control and treated animals (Bornstein et al., 1998). This would suggest that CRHR1 signalling may not have as much of a significant influence on overall glucose homeostasis as would be suggested by the CRHR1 KO mouse study described above, although the lack of additional metabolic parameters included in the former study make comparisons difficult. As with global CRHR KO models, pharmacological antagonism *in vivo* can also be problematic when interpreting results due to the possibility of non-specific or secondary effects of blocking peripheral receptors. Despite the discrepancies in the literature, there is evidence to support either type 1 or type 2 CRH receptors contribute to glucose homeostasis with likely mechanisms also involving direct signalling in the endocrine pancreas.

Observations from the previous chapter (chapter 3) support a role for the CRH family in regulating islet function though the physiological purpose of this is still unclear. Based on previous studies (Drynda et al., 2018) one possible physiological purpose for the effects of CRH on islet function could be a role in mediating the islet adaptation to pregnancy. CRH and urocortin peptides have already been implicated in the modulation of important aspects of pregnancy physiology including the induction of labour and inflammatory pathways associated with parturition (McLean and Smith, 1999; Voltolini et al., 2015; You et al., 2014). Given maternal plasma concentrations of CRH increase exponentially in pregnant women (from mid gestation to term), with the placenta identified as the principal source (Thomson, 2013), it is possible that unidentified endocrine functions of biologically active CRH exist. This may include signalling to pancreatic islets to adapt to the maternal insulin demand during pregnancy. Although placental urocortins have been discovered, limited information regarding the characterisation of these peptides during human or rodent pregnancy is available. Activation of both types of islet CRHRs results in the potentiation of GSIS, a feature of pancreatic islet adaptations during pregnancy and thus warrants further investigation.

4.2 Materials and Methods

4.2.1 Animals

All studies involving regulated procedures on research animals were conducted with approval from the King's College London Animal Welfare and Ethical Review Board and were undertaken in accordance with the United Kingdom Home Office Animals (Scientific Procedures) Act 1986. Mice were housed under controlled conditions (12-hour light (0700-1900) /dark cycle, temperature 22 ± 2^{o}C) and provided with standard chow diet and water *ad libitum*.

All experimental mice used were female CD1 mice (Charles River Laboratories, Harlow, UK), aged between 8 – 14 weeks. The studies described below utilised pregnant and non-pregnant aged matched mice. For timed pregnancy studies, female mice were mated with male CD1 mice and the presence of vaginal plug assessed daily and denoted day 1 of pregnancy if present (under the supervision of biological service unit staff). Procedures were carried out at the same time intervals for non-pregnant animals as described below for pregnancy studies.

4.2.2 RNA extraction and real time qPCR

RNA was extracted from frozen islets isolated from control non-pregnant mice or mice at gestational day 16 (PD.16) along with placenta samples (PD.16), using the RNEasy Mini Kit (Qiagen, UK) as described in section 2.4.1. Once RNA had been quantified and reverse transcribed to cDNA (see section 2.4.1.1 and section 2.4.2), a total of 30 ng cDNA per well was used for real time qPCR reactions (see section 2.4.3.1 for full method). Mouse genes analysed in isolated islets included *Crhr1* and *Crhr2*. Genes analysed in mouse placenta included; *Crh*, *Ucn1*, *Ucn2* and *Ucn3*. Mouse *Gapdh* was used as the housekeeping gene and all genes of interest were normalised to relative expression of this gene. See Table 2-11 for QuantiTect® primer catalogue numbers.

4.2.3 Measurement of circulating CRH peptides

Following euthanasia of control non-pregnant or pregnant (PD.16) CD1 mice, terminal blood samples were collected via cardiac puncture into Eppendorf tubes, pre-coated with heparin (5000 U/ml) (see section 2.6.5 for full protocol). The subsequent plasma collected from centrifugation was used to quantify circulating levels of CRH-like peptides using commercially available ELISA kits as detailed in Table 2-13 according to the manufacturers' instructions.

4.2.4 Subcutaneous implantation of osmotic minipumps (OMPs)

OMPs (ALZET®, Model 1002, Charles River, UK) were subcutaneously implanted on pregnancy day 7 (PD.7) (or equivalent time interval for non-pregnant animals) to chronically administer either non-selective (α-helical CRF_{9-41}, Tocris, UK) or selective (Antalarmin hydrochloride, Tocris, UK; Antisauvagine-30, Tocris, UK) CRHR antagonists (see Table 2.12 and Table 4-2 below). To assess the consequence of total CRHR blockade on glucose homeostasis during pregnancy and outside of pregnancy, α-helical CRF_{9-41}, was used. Antalarmin hydrochloride and Antisauvagine-30 were used to assess the consequences of CRHR1 and CRHR2 blockade, respectively during pregnancy. Surgical implantation of OMPs were carried out as described in section 2.5.1. OMPs infused test agents at a rate of 0.25 µl/ h for a total period of 11 days as per the experimental timeline shown in Figure 4-1.

4.2.4.1 Calculation of approximate circulatory antagonist levels infused by osmotic minipumps

Substances given subcutaneously represent a rapid, inexpensive and simple method of administration to laboratory animals and are generally absorbed at a slower rate providing a sustained effect (Turner et al., 2011). However, infusing agents chronically lends itself to several considerations and challenges when trying to predict the physiological scenario of substances *in vivo*. This is because although the dose administered to the animal is known, achieving complete receptor blockade or sustained desired physiological circulating levels will depend on balancing the concentration and infusion rate of the substance with its clearance rate. The concentration around which the substance/drug concentration consistently stays (i.e. where the rate of infusion of a substance/drug is proportional to the rate of elimination) is known as the steady state concentration. Therefore, according to pharmacokinetic principles, when a drug is continuously administered, provided the infusion rate doesn't change, it will reach steady state after approximately 4 half-lives at which point the plasma concentration will remain consistent (Wadhwa and Cascella, 2020). Below are calculations to determine approximate circulatory levels of each CRHR antagonist at steady state. However, the concentration of antagonists loaded in osmotic minipumps was limited by the maximum solubility of the antagonist in its vehicle and the maximum drug reservoir volume of 100 µl.

Pharmacokinetic metric	Symbol	Description
Steady state concentration	C_{ss}	The time during which the concentration remains stable or consistent when the drug is given repeatedly or continuously
Infusion rate	K_{in}	Rate of infusion required to balance elimination
Clearance	CL	The volume of plasma cleared of the drug per unit time
Volume of distribution	V_d	The volume in which a drug is distributed
Elimination rate constant	K_e	The rate at which a drug is removed from the body
Elimination half life	$t_{1/2}$	The time required for the concentration of the drug to reach half of its original value

Table 4-1 Description of pharmacokinetic principles applied for steady state calculation

CRHR Antagonist	Concentration loaded in OMP	Amount of antagonist delivered per hour (i.e. K_{in} -Infusion rate)
α-helical CRF$_{9-41}$ (M. wt: 3827)	1 mg/ml (261μM)	65.25 pmoles/h
Antalarmin hydrochloride (M. wt: 415)	1 mg/ml (2.4 mM)	600 pmoles/h
Antisauvagine-30 (M. wt: 3650)	3 mg/ml (822 μM)	205.5 pmoles/h

Table 4-2 Infusion rate of each CRHR antagonist in osmotic minipump

Steady state concentration equation: $\mathbf{C_{ss} = K_{in} / CL}$

CL, K_e, V_d, and $t_{1/2}$ are all inter-related as follows:

$K_e = CL / V_d$ $t_{1/2} = 0.693 / K_e$ therefore, $K_e = 0.693 / t_{1/2} = CL / V_d$

To solve for clearance *(CL):* $CL = V_d$ x $(0.693 / t_{1/2})$

The average total blood volume in a mouse is ~3 ml (0.003 l)

Biological half-lives reported for α-helical CRF$_{9-41}$, Antalarmin hydrochloride and Antisauvagine-30 are 0.5 h, 1.5 h and 1 h respectively (Chen et al., 1997; Saphier et al., 1992; Wiley and Davenport, 2004).

Therefore, the clearance of:

α-helical CRF$_{9-41}$ = 0.003 l x (0.693 / 0.5 h) >> CL = 0.004 l/ h

Antalarmin hydrochloride = 0.003 l x (0.693 / 1.5 h) >> CL = 0.0014 l/ h

Antisauvagine-30 = 0.003 l x (0.693 / 1 h) >> CL = 0.002 l/ h

Steady state concentrations (C_{ss}) of:

α-helical CRF_{9-41} = 65.25 pmoles/h / 0.004 l/h >> **16.31 nmoles/l**

Antalarmin hydrochloride = 600 pmoles/h / 0.0014 l/h >> **428 nmoles/l**

Antisauvagine-30 = 205.5 pmoles/h / 0.002 l/h >> **102.75 nmoles/l**

***Nb**: These circulating concentrations are expected to achieve full receptor blockade based on the reported EC50/IC50 values for the receptors* (Rivier and Rivier, 2014; Seymour et al., 2003).

4.2.5 Metabolic glucose and insulin tolerance testing *in vivo*

Glucose clearance and insulin sensitivity was assessed in mice via an intraperitoneal glucose tolerance test (GTT) (2 g/kg) and insulin tolerance test (ITT) (0.75 IU/kg) conducted on PD.16 and PD.18 respectively. Equivalent time intervals were employed in non-pregnancy studies. Mice were fasted from 0900 for 6 hours and weighed prior to metabolic testing, as previously described in section 2.6.1 and section 2.6.2. Accu-Chek glucose meter and strips (Roche Diagnostics, UK) were used for the determination of blood glucose levels (sampled from a small tail prick) at set time intervals over a 2-h or 1-h time course for GTT and ITT respectively. During the course of the GTT, additional blood sampling was carried out at baseline (0 min) and 30 min post glucose administration for the subsequent measurement of plasma insulin (described in section 2.6.1.1) using a commercial mouse insulin ELISA (10-1247-01, Mercodia, Sweden) following the manufacturer's instructions.

4.2.6 Bromodeoxyuridine (BrdU) administration and terminals

BrdU (1 mg/ml) (Sigma, UK) was administered in the drinking water from gestational day 14 (PD.14) till day 18 (or equivalent time intervals in non-pregnant mice) for the assessment of proliferating cells *in vivo*. The BrdU-containing drinking water was replaced every two days. At the end of the study (after ITT on PD.18), mice were euthanised via terminal anaesthesia and pancreata were dissected and fixed in 4% formaldehyde (Sigma, UK) for 48 h prior to wax embedding (see section 2.7.1) and subsequent immunohistochemistry (see section 2.7.3).

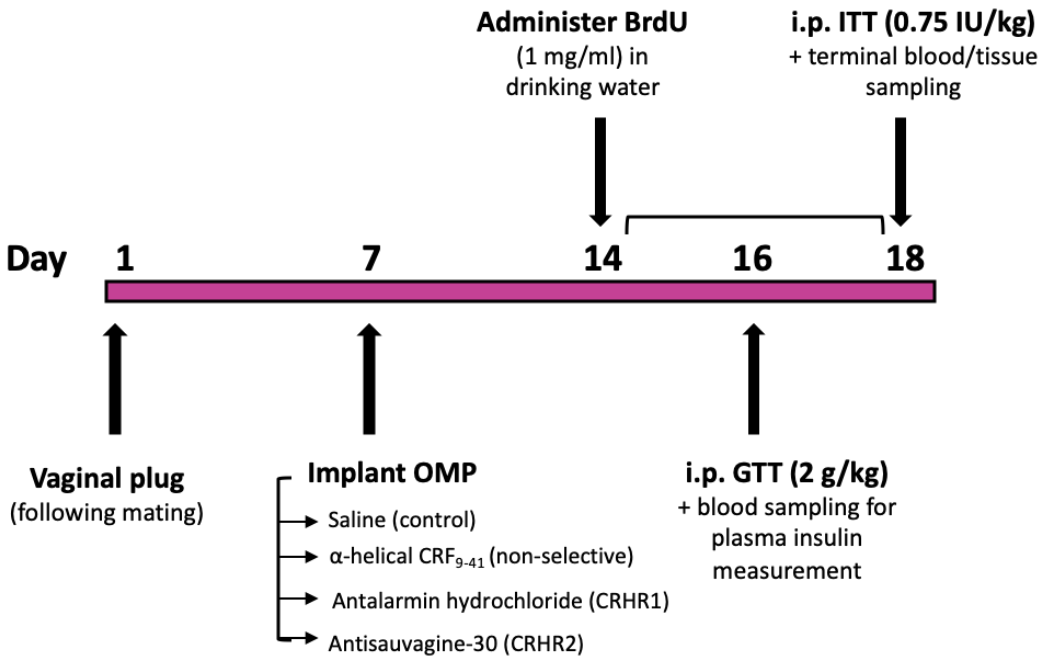

Figure 4-1 Experimental timeline for OMP studies

4.2.7 Immunohistochemistry

Paraffin wax embedded sections (5 μm thick) were stained against insulin and BrdU, using guinea pig polyclonal anti-insulin antibody (1:200) (Dako, UK) and mouse monoclonal anti-BrdU antibody (1:100) (Sigma, UK) respectively (as described in section 2.7.3). Fluorescently labelled secondary antibodies, Alexa-Fluor 488 and Alexa-Fluor 594 (both 1:50) (Jackson, UK) were used to visualise BrdU and insulin staining respectively. Sections were imaged using a Nikon Eclipse TE2000-U microscope and NIS elements software and morphological analysis conducted using ImageJ software as described in section 2.7.4.

4.3 Results

4.3.1 Characterisation of islet CRHR and placental CRH/UCN peptide gene expression and circulating profile during mouse pregnancy

We and others have reported mRNA expression of CRH receptors (CRHR) in mouse islets however, their expression pattern in islets during pregnancy still remains relatively uncharacterised. As shown in Figure 4-2, CRHR mRNA for both *Crhr1* and *Crhr2*, was expressed in islets isolated from non-pregnant and pregnant (PD.16) female mice. *Crhr1* expression was approximately 10-fold higher than *Crhr2* expression in non-pregnant control islets, consistent with our findings from the previous results chapter (chapter 3). By day 16 of pregnancy however, *Crhr1* expression was significantly reduced (~60%) compared to non-pregnant levels (Figure 4-2A), whereas islet *Crhr2* expression levels remained unchanged between the non-pregnant and pregnant state (Figure 4-2B).

Previous studies have demonstrated expression of CRH related peptides in the placenta (Drynda et al., 2018). Real-time qPCR analysis confirmed mRNA expression of *Crh*, *Ucn1*, *Ucn2* and *Ucn3* in mouse placenta on day 16 of pregnancy at similar levels ($p = 0.1503$) as shown in Figure 4-2C. To investigate whether detectable peptide levels were found in the circulation during pregnancy, terminal plasma samples from non-pregnant and pregnant (PD.16) mice were assayed. All four peptides were detected in both states, ranging from 2 pM – 93 pM in non-pregnant mice and 4 pM – 178 pM in pregnancy. Although circulating levels of CRH, UCN1 and UCN3 were unchanged between non-pregnant and pregnant animals, UCN2, the most abundant circulating CRHR agonist, significantly increased (~92%) by PD.16 when compared to age-matched non-pregnant female controls (Figure 4-2D).

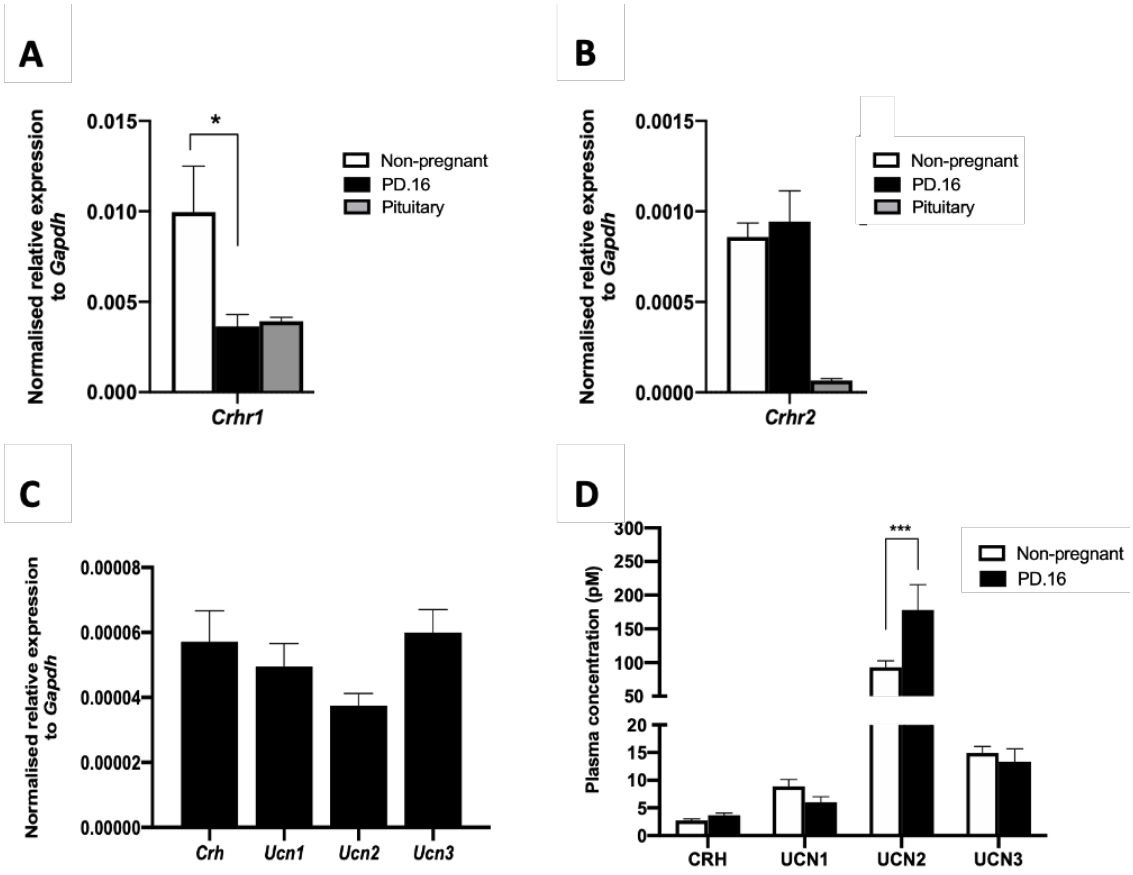

Figure 4-2 Characterisation of islet CRHR and placental CRH/UCN peptide gene expression and circulating profile during mouse pregnancy. mRNA expression levels of *Crhr1* (A) and *Crhr2* (B) were measured in isolated female islets from non-pregnant (white bar) and pregnant day.16 (PD.16) (black bar) mice. Pituitary samples (grey bar) were used as a positive control. mRNA expression levels of CRH and UCNs were measured in PD.16 mouse placenta (C). Expression levels were normalised relative to expression of housekeeping gene, *Gapdh.* Plasma levels of all four peptides were also quantified (D) in non-pregnant female mice (white bar) and compared to pregnant levels on PD.16 (black bar). Data are presented as mean ± SEM, n = 5-6, **p* < 0.05 (non-pregnant vs PD.16), Two tailed t-test or one-way ANOVA for placental ligands. Plasma data (D); n= 6-8, ***p < 0.001, two-way ANOVA followed by Tukey's multiple comparison test.

4.3.2 Effects of pharmacologically blocking total endogenous CRHR signalling on glucose homeostasis during pregnancy

Chronic administration of a non-selective CRHR antagonist (α-helical CRF_{9-41}) via subcutaneously implanted OMPs was used to assess the consequences of blocking endogenous ligands for both CRHR1 and CRHR2 on glucose homeostasis during pregnancy. OMPs were implanted on PD.7, 9 days prior to scheduled GTT on PD.16 where peak rodent β-cell adaptations occur (Rieck and Kaestner, 2010). Intraperitoneal administration of glucose elevated blood glucose levels within 15 mins for both control and α-helical CRF_{9-41} treated mice as shown in Figure 4-3A. Blockade of total CRHR signalling resulted in a mild impairment to glucose tolerance, with significantly higher blood glucose concentrations at 15 minutes post glucose load, compared to saline controls (α-helical $CRF_{9–41}$ vs Control; 16.83 ± 1.60 vs 13.22 ± 1.08 mM, **p<0.01) (Figure 4-3A). This impairment was transient as by 30 mins, glucose tolerance had returned to similar levels to that of control mice, which is also represented by the total glucose AUC for both 0-30 min and 0-120 min (Figure 4-3B).

Although both groups exhibited similar fasting plasma insulin, measurement of plasma insulin in response to glucose administration revealed that pregnant mice treated with α-helical $CRF_{9–41}$, displayed an ~18% decrease in GSIS in comparison to pregnant controls (α-helical $CRF_{9–41}$ vs Control: 30 min; 192.14 ± 37.93 vs 235.13 ± 28.14 pM). However, this did not reach statistical significance (Figure 4-3C). By day 16 of pregnancy, no differences in body weight were observed between control and CRHR antagonist groups both reaching an average weight of 50 g (Figure 4-3D). All pregnant mice were insulin resistant by late pregnancy (PD.18) as indicated by the failure to respond to exogenous insulin administration and lowering of blood glucose shown in Figure 4-3E. Chronic treatment with α-helical $CRF_{9–41}$ did not appear to have any detectable effects on insulin sensitivity with similar AUC calculated for the duration of the insulin tolerance test (0-60 min) for both groups (Figure 4-3F).

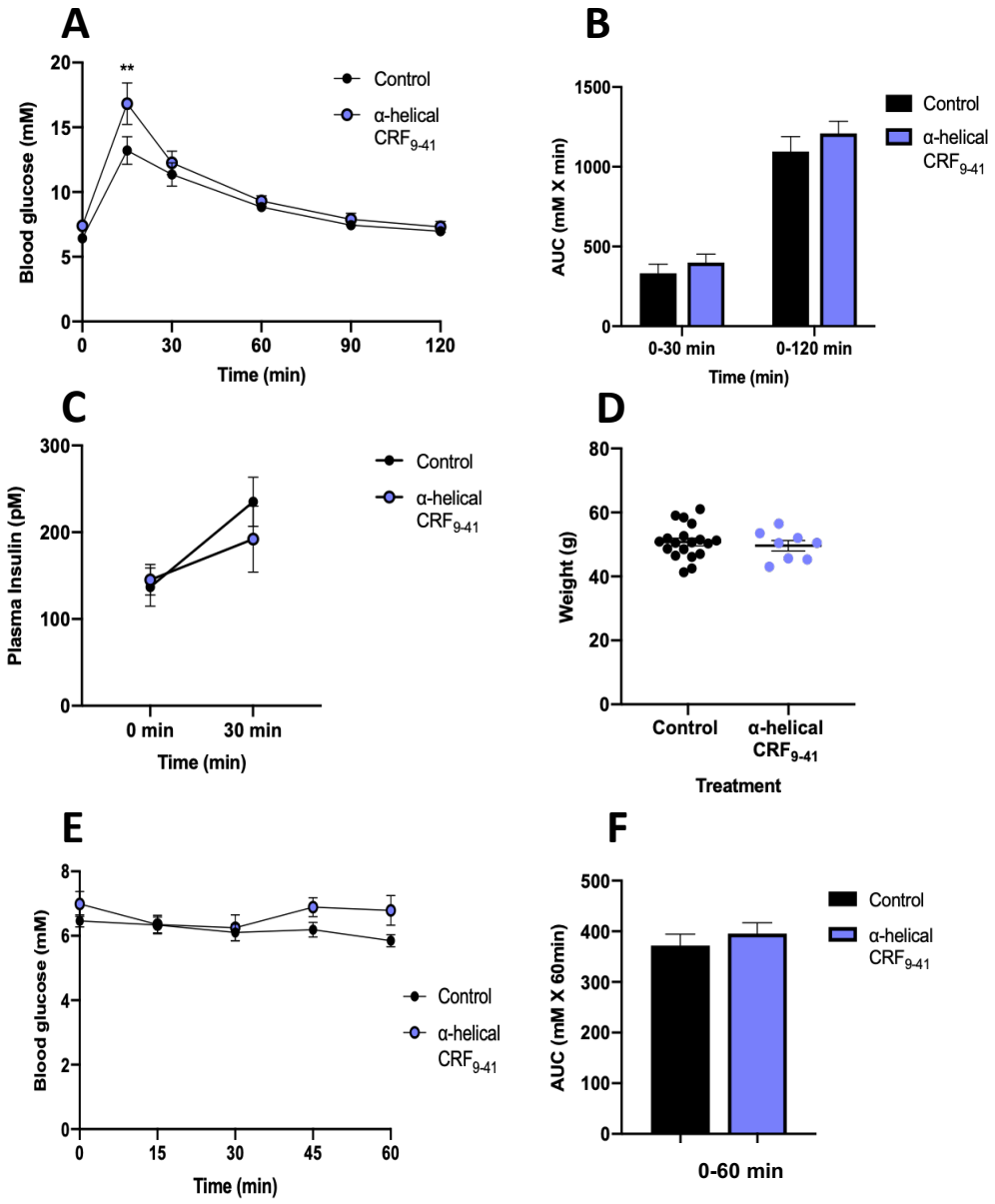

Figure 4-3 Effects of chronic administration of a non-selective CRHR antagonist on glucose homeostasis during pregnancy. Mice implanted with OMPs containing control (saline) (black circle/bar) or non-selective CRHR antagonist (α-helical CRF_{9-41}) (lilac circle/bar) on gestational day 7 (PD.7), underwent a fasted i.p. GTT on PD.16 (A). Representative glucose AUC for both 30 mins post glucose administration (0-30 min) and the entire test (0-120 min) are displayed (B). GTT fasting/baseline and 30 min post glucose administration plasma insulin (C) and body weight (PD.16) (D). Fasted i.p. ITT (PD.18) (E) and representative glucose AUC for entire test (0-60 min) (F) for both groups. Data are presented as mean ±SEM, n= 8-19 mice/group, $^{**}p<$ 0.01 two-way RM-ANOVA followed by Sidak's multiple comparisons test.

4.3.3 Effects of pharmacologically blocking selective CRHR signalling on glucose homeostasis during pregnancy

To elucidate the effects of endogenous signalling through CRHR1 and CRHR2 independently during pregnancy, selective CRHR antagonists were chronically infused in pregnant mice as described above in section 4.2.4. Antalarmin hydrochloride (AH - selective against CRHR1) and Antisauvagine-30 (AS-30 -selective against CRHR2) were systemically administered (via OMPs) for 9 days prior to scheduled GTT on day 16 of pregnancy alongside pregnant saline control mice. All mice displayed a rapid increase in blood glucose levels at 15 mins following intraperitoneal injection of glucose (Figure 4-4A). However, mice administered the CRHR2 antagonist, Antisauvagine-30, displayed impaired glucose tolerance at 15 mins post glucose administration, with significantly higher glucose levels compared to both control and CRHR1 selective antagonist treatment (AS-30 vs Control; 16.63 ± 1.60 vs 13.22 ± 1.08 mM, *$p<0.05$; AS-30 vs AH; 16.63 ± 1.60 vs 11.93 ± 1.33, **$p<0.01$) (Figure 4-4A). Similar to results with the non-selective CRHR antagonist, by 30 mins all groups had returned to similar glucose levels as represented by the calculated glucose AUC (0-30 min) shown in Figure 4-4B. The glucose profile thus mirrored that of control animals for the remainder of the GTT. In response to glucose administration, all treatment groups displayed a similar increase in plasma insulin levels, increasing by approximately 42%, 46% and 42% for control, AH and AS-30 treated animals respectively (Figure 4-4C) although fasted and GSIS was lowest for the AS-30 group (Control: 0 min vs 30 min; 136.95 ± 22.03 vs 235.13 ± 28.14 pM; AH: 118.15 ± 46.16 vs 218.99 ± 50.79; AS-30: 113.90 ± 31.69 vs 196.23 ± 33.07). By day 16 of pregnancy, no differences in gestational weight were observed between treatment groups as shown in Figure 4-4D. Chronic treatment with either CRHR antagonist did not appear to have any detectable effects on insulin sensitivity (Figure 4-4E &F) or the number of pups per litter (Figure 4-4G).

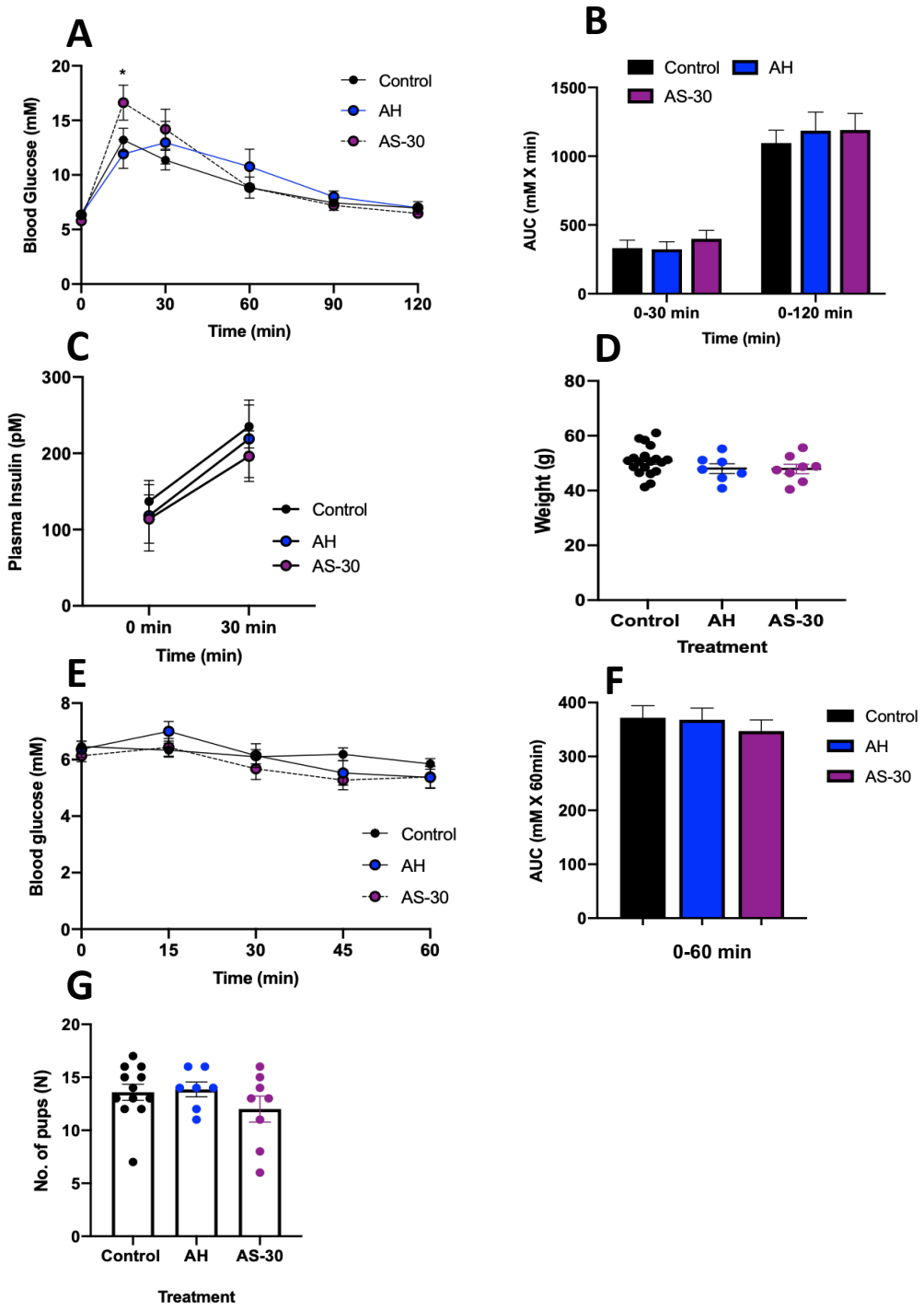

Figure 4-4 Effects of chronic administration of selective CRHR antagonists on glucose homeostasis during pregnancy. Mice implanted with OMPs containing either control (saline) (black circle/bar), Antalarmin hydrochloride (AH) (blue circle/bar) or Antisauvagine-30 (AS-30) (plum circle/bar) on PD.7, underwent a fasted i.p. GTT on PD.16 (A). Representative glucose AUC for both 30 mins post glucose administration (0-30 min) and the entire test (0-120 min) are displayed (B). GTT fasting/baseline and 30 min post glucose administration plasma insulin (C) and body weight (PD.16) (D). Fasted i.p. ITT (PD.18) (E) and representative glucose AUC for entire test (0-60 min) (F) for all treatment groups. Animals were euthanized on PD.18 following ITT and the number of pups (G) were recorded. Data are presented as mean ±SEM, n= 7-19 mice/group, *$p<$ 0.05 two-way RM-ANOVA or one-way ANOVA followed by Tukey's multiple comparisons test.

4.3.4 Effects of blocking endogenous CRHR signalling on β-cell proliferation and morphology during pregnancy

Pregnancy-induced pancreatic β-cell adaptions include changes to the proliferative capacity and morphology to expand the pool of functional insulin secreting β-cells. Therefore, as per the experimental timeline (Figure 4-1), BrdU was administered in the drinking water of pregnant mice to assess proliferating cells *in vivo*. To investigate the maximal effect of total CRHR antagonism, pancreata dissected from mice chronically administered the non-selective CRHR antagonist (α-helical $CRF_{9–41}$) were immunoprobed for both BrdU and insulin. No differences in staining or islet morphology were observed upon visualisation of islets from either saline treated controls or α-helical $CRF_{9–41}$ (as indicated by the representative images of each condition in Figure 4-5A & B). Analysis of the percentage of β-cells that were BrdU positive revealed no significant effects on β-cell proliferation (Control vs α-helical $CRF_{9–41}$; 3.75 ± 0.56 vs 4.50 ± 0.55 %, p=0.357) (Figure 4-5C). β-cell size or the overall $insulin^+$ islet area was also unchanged with chronic α-helical $CRF_{9–41}$ treatment as shown in Figure 4-5D & E.

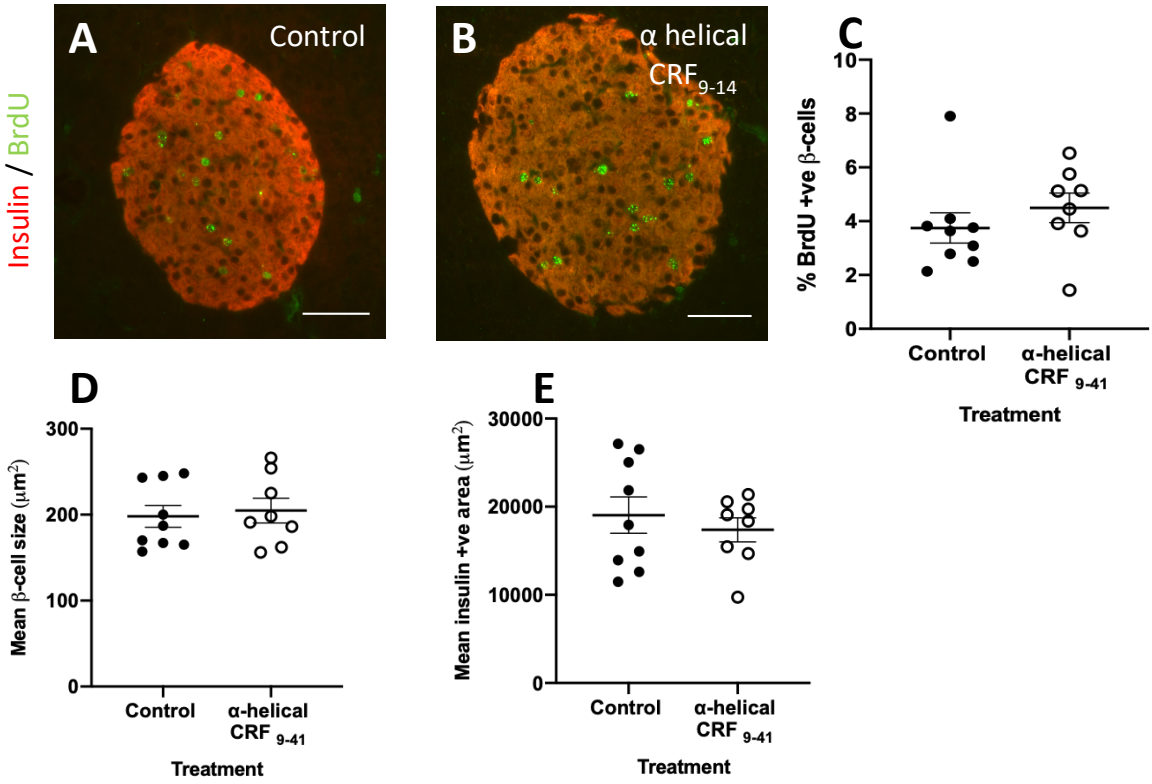

Figure 4-5 Effect of chronic administration of a non-selective CRHR antagonist on β-cell morphology during pregnancy. Mice were administered BrdU-containing drinking water from days 14–18 of pregnancy that were either treated with saline (control) (black circles) or α-helical $CRF_{9–41}$ (white circles) from PD.7. Representative images of immunostaining for the measurement of β-cell proliferation in control (A) and α-helical $CRF_{9–41}$ (B) islets showing insulin staining (red) and BrdU staining (green). Percentage of BrdU-labelled β-cells (C), mean β-cell size (D) and mean β-cell islet area (E) are shown. Data are presented showing quantification of 3-4 sections per animal with bar representing mean ±SEM, n= 8-9 animals per treatment group. Statistical significance analysed with two-tailed t-test. Scale bar 50 μm.

4.3.5 Consequence of pharmacologically blocking total CRHR signalling in non-pregnant mice

To investigate whether the effects observed with chronic blockade of CRHR signalling on whole body glucose homeostasis were confined to pregnancy, OMPs loaded with α-helical $CRF_{9–41}$ were implanted into female non-pregnant mice. Following the equivalent experimental timeline as for pregnancy studies, metabolic testing to assess glucose clearance following 9 days of infusion revealed no significant differences in the glucose tolerance between saline controls or animals treated the CRHR antagonist (Figure 4-6A & B). No statistically significant differences were displayed in plasma insulin at either baseline or 30 mins after i.p. administration of glucose with both conditions exhibiting similar responses to GSIS (30 min) of 40% and 47% increase for controls and α-helical $CRF_{9–41}$, respectively (Figure 4-6C). Body weight and insulin sensitivity were also similar between both groups (Figure 4-6D, E & F). Given the lack of effect of α-helical $CRF_{9–41}$, receptor-specific antagonists were not tested outside of pregnancy.

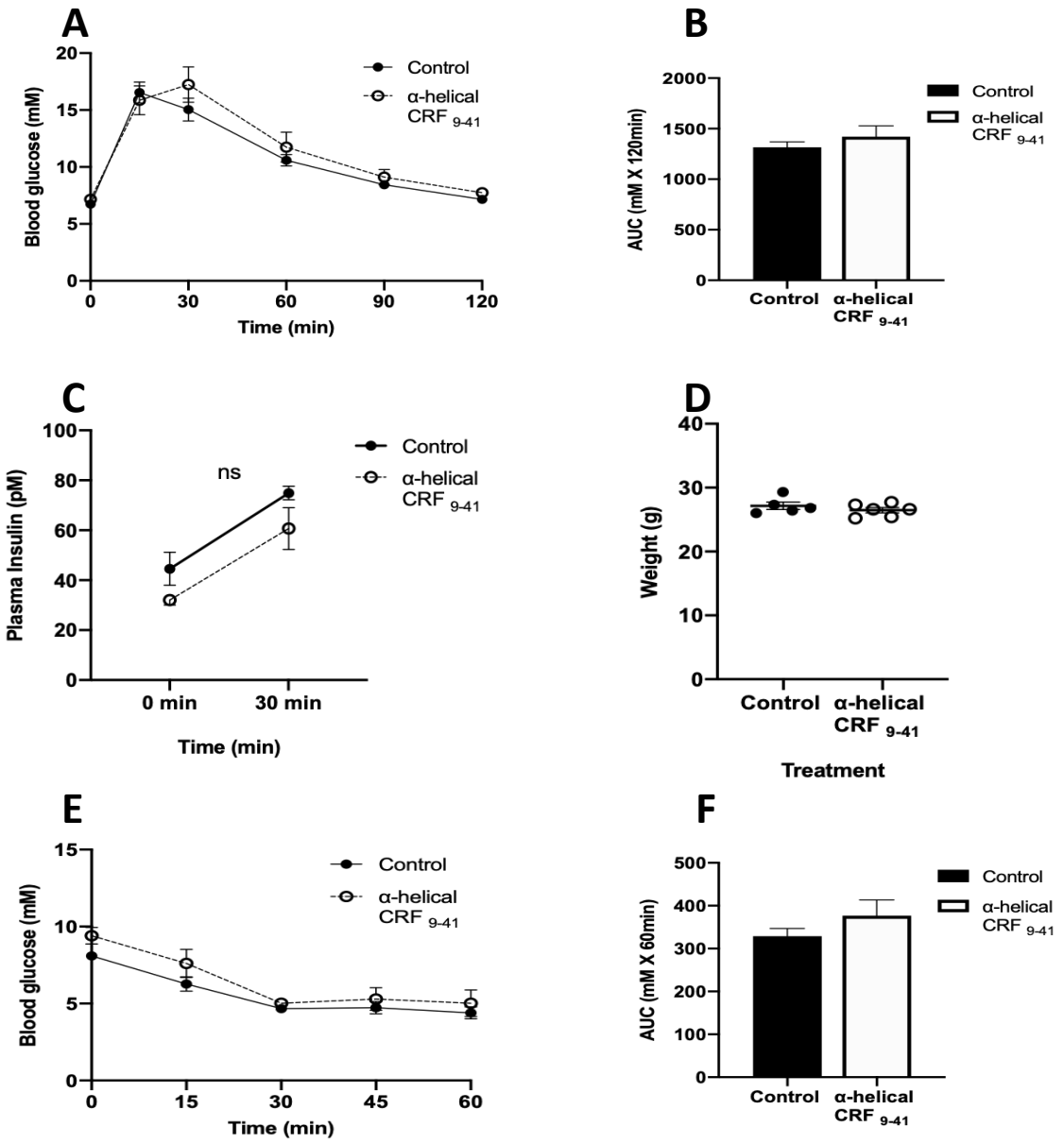

Figure 4-6 Effect of chronic administration of a non-selective CRHR antagonist on glucose homeostasis in non-pregnant mice. Mice implanted with OMPs containing saline (control) (black circle/bar) or non-selective CRHR antagonist (α-helical CRF_{9-41}) (white circle/bar) on gestational day 7 (PD.7), underwent a fasted i.p. GTT on PD.16 (A). Representative glucose AUC for the entire test (0-120 min) are displayed (B). GTT fasting/baseline and 30 min post glucose administration plasma insulin (C) and body weight (PD.16) (D). Fasted i.p. ITT (PD.18) (E) and representative glucose AUC for entire test (0-60 min) (F) for both groups. Data are presented as mean ±SEM, n= 5-6 mice/group. Statistical significance analysed using two-way RM-ANOVA followed by Sidak's multiple comparisons test.

4.4 Discussion

Pregnancy is characterised by a physiological increase in maternal insulin resistance, representing a key mechanism for increasing fuel availability to the fetus (Freemark, 2006; Newbern and Freemark, 2011). The alterations in maternal metabolic profile places unique demands on the pancreatic β-cells, which undergo a series of regulated alterations to morphology and function to compensate for the metabolic pregnant environment (Ernst et al., 2011; Plows et al., 2018). Maternal normoglycaemia is therefore maintained by an expanded, hyperfunctional β-cell population. Disturbances to these adaptive mechanisms can lead to maternal glucose intolerance and possibly overt gestational diabetes (Plows et al., 2018). Understanding the regulators and mechanisms underlying gestational β-cell adaptations using animal models may unravel networks involved in the crosstalk between the maternal pancreas and placenta.

Lactogenic signalling via β-cell-specific PRLRs has become a well-established mechanism (at least in rodents) for inducing β-cell mass expansion and enhanced insulin secretion during pregnancy (Huang et al., 2009; Sorenson and Brelje, 2009). Subsequent research has since implicated β-cell-serotoninergic pathways downstream of PRLR activation mediating, to some extent, these β-cell-specific adaptations (Kim et al., 2010; Ohara-Imaizumi et al., 2013). However, given the significant alterations to the maternal hormonal milieu during pregnancy, with the mouse placenta expressing mRNA for 79 different endogenous ligands for which β-cells express the cognate GPCR (Drynda et al., 2018), it is likely that other signals are also involved in regulating pregnancy-induced islet adaptations. Despite, the potential interactions between these placenta ligands and islet GPCRs, only a few have been investigated for possible effects during pregnancy. More recently, studies into other placental signals aside from steroid hormones, including; kisspeptin, adiponectin, hepatocyte growth factor (HGF) and leptin have been implicated in regulating the islet adaptation to pregnancy and the development of GDM (Simpson et al., 2018). These placental peptides are released at high levels during pregnancy from the placenta and although they are more commonly linked to effects on reproduction, insulin sensitivity, cell growth and food intake respectively, accumulating evidence supports additional direct effects on pancreatic β-cells. For example, insulinotropic effects of kisspeptin and adiponectin have be demonstrated *in vitro* and *in vivo* in rodents (Bowe et al., 2009; Cantley, 2014; Hauge-Evans et al., 2006; Okamoto et al., 2008). Pregnant adiponectin knockout mice or kisspeptin receptor antagonism specifically during pregnancy both reveal impaired islet adaptations including insulin deficiency and reduced β-cell mass (Bowe et al., 2019; Qiao et al., 2017). Clinical studies have also revealed decreased serum levels of both peptides in GDM compared with healthy pregnancies (Bowe et al., 2019; Ćetković et al., 2012; J et al., 2014; Pala et al., 2015). The pregnancy and β-cell-specific effects of HGF and leptin are less clear but there is evidence to suggest that raised placental and/or serum levels of these peptides are associated

with increased GDM risk and therefore they may represent useful biomarkers for GDM generally (Bao et al., 2015; Dishi et al., 2015; Simpson et al., 2018). These newly identified placental peptides offer promising therapeutic and/or diagnostic potential however, a clearer understanding of their involvement as well as identifying other novel signals and mechanisms that regulate the islet adaptations to pregnancy are necessary. The *in vivo* studies in this chapter implicates a role for direct CRHR signalling to add to these previously identified mechanisms. Specifically, placental UCN2 acting through islet CRHR2 to control β-cell function and thus maternal glucose tolerance specifically during pregnancy.

Interestingly, the CRHR system within the islet microenvironment appears to be influenced by pregnancy as decreased expression levels of CRHR1 were observed during mouse gestation suggesting a physiological role for CRHR signalling in modifying islet function and/or morphology. The reduced expression of CRHR1 is surprising as a receptor that is actively involved in the islet response to pregnancy might be expected to increase in levels during gestation. However, the fact that CRHR1 levels are significantly altered suggests some physiological role. It is possible that the reduction in CRHR1 expression and maintenance of CRHR2 expression shifts the ratio of islet CRH receptors towards CRHR2 during pregnancy. This is potentially a mechanism to prioritise CRHR2 signalling during pregnancy under the influence of placental signals.

The placenta is a major source of hormones secreted into the maternal circulation informing maternal tissues, including the pancreatic islets, of the gestational status. Therefore, placentally released biomolecules which have been established to influence the endocrine capacity of islets serve as attractive targets for potential involvement in the islet response to pregnancy. In the current study, we detected the expression of mRNAs for all members of the CRH family in the mouse placenta, consistent with prior experimental studies conducted within the group (Drynda et al., 2018). Our results are also in accordance to a number of studies demonstrating detectable peptide mRNA or protein expression in mouse and human placenta (Imperatore et al., 2006; Petraglia et al., 2010; Voltolini et al., 2015, 2012). Together, these data support the idea that these peptides may be involved in the crosstalk between placenta and islet β-cells.

Placental expression of CRH in rodents has been controversial in the literature. In humans, immunoreactive CRH is well known to be expressed and released by the placenta into the maternal circulation (Petraglia et al., 1987; Saijonmaa et al., 1988; Sasaki et al., 1988; Shibasaki et al., 1982). Human pregnancy is also characterised by a dramatic rise in maternal plasma levels of CRH as gestation progresses towards term, peaking during labour (Campbell et al., 1987; McLean et al., 1995; Sasaki et al., 1987). This maternal CRH is of placental origin and is well established in humans as representing a “placental clock”, controlling the length of pregnancy

(Grino et al., 1987b; McLean et al., 1995; Rosen et al., 2015; Saijonmaa et al., 1988). However, production of CRH by the placenta is largely believed to be unique to humans and non-human primates (Robinson et al., 1989; Smith, 2007). Therefore, it has been suggested that despite the extensive conservation of the hypothalamic CRH system in vertebrates, CRH is absent from the placental mechanisms controlling pregnancy and labour in other placental mammals such as rodents (Grammatopoulos, 2008; Robinson et al., 1989). Consequently, very few studies have focused investigations on the role of CRH or CRH related peptides in rodent pregnancy physiology. Only two reports to date have described mRNA expression of CRH in murine placenta in addition to all three urocortin peptides as we have also shown in this study (Drynda et al., 2018; Voltolini et al., 2012). Notably, placental expression levels of the CRH peptides are fairly low which may provide an explanation for discrepancies in the literature, especially earlier studies using less sensitive techniques to detect placental CRH.

Although mRNA transcript levels are a good indicator of corresponding protein levels, not all the time do both correlate. Despite the placental mRNA expression of CRH ligands, our plasma analysis revealed that circulating levels of CRH, UCN1 and UCN3 were unchanged in pregnant mice compared to non-pregnant mice suggesting that these ligands are not released by the mouse placenta at significant levels. However, circulating levels of UCN2 were significantly increased during gestation. This finding is consistent with previous data from Voltolini and colleagues who reported an increase in mouse placental UCN2 during gestation (Voltolini et al., 2015) suggesting CRH-like peptides could function as endocrine mediators within the systemic circulation during pregnancy. Characterisation of the binding properties and functional activities of CRH family ligands in Chinese hamster ovary cells stably transfected with either human CRHR1 or murine CRHR2, revealed inhibitory binding constants (Ki) for mouse UNC2 of 2.1 nM and 0.66 nM for CRHR2α and CRHR2β respectively (Lewis et al., 2001b). Additionally, cAMP assays (conducted as a measure of cellular activation and thus ligand potency), revealed EC50 values (half maximal effective concentration) of 0.14 nM and 0.05 nM for CRHR2α and CRHR2β respectively (Lewis et al., 2001b). Although the β-cell specific CRHR2 isoform is yet to be confirmed, it is widely accepted that in rodents, the predominant peripheral isoform is CRHR2β (Grammatopoulos and Chrousos, 2002; Lovenberg et al., 1995a). Therefore, it is reasonable to assume that the circulating concentration of UCN2 which we detected during pregnancy (~200 pM) is capable of binding and activating β-cell CRHR2, given the closeness to the reported Ki and EC50 values. This suggests that endogenously activated β-cell CRHR2 by elevated maternal UCN2 may play a role in regulating islet responses supported by the observation from the previous chapter that either CRHR subtype can enhance insulin secretion (chapter 3). The placental and circulatory profile of CRHR ligands, particularly the increase in circulatory UCN2, is also consistent with the placenta being the source of the elevated levels of UCN2 during mouse pregnancy. However, it cannot be ruled out that the pregnancy-associated UCN2 derives from an alternative peripheral source, such

as skin or skeletal muscle where it is also highly expressed, (Chen et al., 2004) under the influence of other pregnancy signals.

Irrespective of its source, the profile of pancreatic CRH receptors and circulating ligands in response to pregnancy supports a potential novel role for CRHR signalling in regulating β-cell physiology during gestation. In order to investigate this hypothesis, we used subcutaneously implanted osmotic minipumps to chronically administer CRHR antagonists to assess the consequence of blocking endogenous CRHR signalling/ligands on maternal glucose homeostasis. Continuous drug infusion via osmotic minipump allowed for an alternative approach to frequent animal injections and handling and thus enabled minimal repetitive stressors for the mice throughout the sensitive gestational period.

Pharmacological blockade of total CRHR signalling, using α-helical CRF_{9-41}, a non-selective CRHR antagonist, resulted in a mild and transient impairment to glucose tolerance in pregnant mice. This impaired glucose tolerance was associated with a decrease in glucose-induced insulin secretion without causing any detectable effects on insulin sensitivity. This provided initial indication that an endogenous ligand, signalling via either CRHR1 or R2 may be responsible for maintaining maternal glucose tolerance during pregnancy. Receptor selective antagonists were able to elucidate that this endogenous ligand is selectively signalling via CRHR2, as glucose tolerance was impaired by selective pharmacological blockade of CRHR2 (using Antisauvagine-30) but not with CRHR1 blockade (using Antalarmin hydrochloride). Although a relatively modest decrease in plasma insulin was displayed with Antisauvagine-30 treatment in comparison to that observed with α-helical CRF_{9-41}, the impairment appears to reflect a β-cell targeted effect, as no measurable changes to insulin sensitivity were observed. Alterations in glucose tolerance were independent of any changes in body weight, as gestational weight gain and litter size were unaltered between treatment groups. Given CRHR expression has been reported in key target insulin tissues including skeletal muscle, adipose tissue and the liver (Lovenberg et al., 1995a; Paschos et al., 2013; Sakamoto et al., 2013; Seres et al., 2004; Simopoulos et al., 2009), it may have been expected that any potential off-target/non-specific effects from endogenous CRHR blockade might also alter insulin signalling and thus overall insulin sensitivity. *In vitro* evidence in skeletal muscle cells has also revealed increased glucose uptake (i.e. increased insulin sensitivity) following CRHR2 activation (Gao et al., 2016) which would suggest that if this signalling pathway was blocked, insulin sensitivity in the mice may decline. However, no detectable effects on insulin sensitivity were observed following *in vivo* CRHR blockade but this may have been because the mice were already insulin resistant, attributable to the pregnancy environment. Although a higher dose of insulin might have been necessary to circumvent the pregnancy-associated insulin resistance, this may come at a greater risk of a maternal hypoglycaemic event which would also be undesirable to the foetuses. One may also speculate that if potential mechanisms of the glucose tolerance observed with

CRHR2 blockade were as a result of alterations to insulin sensitivity then a more pronounced and sustained impairment to glucose tolerance may have been observed. Nonetheless, CRHR blockade in non-pregnant mice with normal insulin sensitivity still displayed no alterations in insulin tolerance. This supports the hypothesis that the effects of CRHR2 blockade on glucose homeostasis are through stopping the activation of islet-specific CRHR2 altering β-cell function and reducing insulin secretion. Based on our previous observations of changes in circulating levels of the CRH family during pregnancy, the endogenous ligand mediating these effects is most likely UCN2.

Our *in vitro* data, demonstrating the insulinotropic effects of islet CRHR activation, is consistent with these *in vivo* studies and the theory that signals directed towards β-cell CRHR2 support functional β-cell adaptive responses to pregnancy. However, an important detail to consider is the relative contribution of blocking CRHR2 on other islet cells as expression of type 2 CRHRs by islet delta cells has been reported (Rorsman and Huising, 2018; van der Meulen et al., 2015). Although UCN3, another endogenous CRHR2 selective ligand, has been shown to stimulate insulin secretion (Li et al., 2003), local UCN3 signalling on delta cells has also been shown to regulate somatostatin-dependent negative feedback control of insulin secretion (van der Meulen et al., 2015). Therefore, whilst selective CRHR2 blockade may directly reduce insulin secretion from the β-cells it may also have opposing effects by reducing somatostatin release from δ-cells and indirectly increasing insulin release. This may explain why a more pronounced reduction in plasma insulin was not observed in response to Antisauvagine-30. Despite this, it appears that the net effect of CRHR2 antagonism is a reduction in insulin secretion via β-cell-mediated mechanisms. The generation of a conditional β-cell-specific CRHR2 knockout (CRHR2 KO) mouse would allow for more precise characterisation and validation of the direct β-cell specific CRHR2 signalling mechanisms in the islet adaptations to pregnancy. This would eliminate any off-target effects which could be influencing the metabolic profile of these mice. Until recently, only constitutive CRHR2 KO mice had been established (Keck et al., 2005). Mice harbouring a floxed *crhr2* allele now exist allowing for the generation of conditional CRHR2 KO mice which have since been used for example, in studies investigating CRHR2 deficiency in cardiomyocytes and serotonergic neurons (Gracie, 2015; Tsuda et al., 2017). Therefore, there is the future potential to, using cre-loxP recombinase technology, under the control of a β-cell specific promoter gene (e.g. mouse insulin 1 promoter (MIP), which drives cre recombinase activity exclusively in the β-cell) (Smith et al., 2020), to study the effects of an inducible β-cell CRHR2 KO during pregnancy. This approach has successfully been used in the study of placental kisspeptin and islet adaptations to pregnancy in the β-cell specific GPR54 knock down mouse line (Bowe et al., 2019).

With various members of the CRH peptide family detected in key peripheral metabolic tissues (e.g. skeletal muscle) (Kuperman and Chen, 2008) a role for peripheral CRHR signalling in

overall glucose/energy homeostasis has previously been investigated, although not generally with a focus on islet function. One such study involved pharmacological CRHR2 blockade *in vivo* with Astressin 2B (an alternative synthetic CRHR2-selective antagonist) in male rats and demonstrated a slight worsening of glucose tolerance compared to vehicle control treatment. This impairment also mirrored the attenuation to glucose-induced insulin secretion *in vivo* with treatment of the antagonist (Li et al., 2007). These results are generally consistent with the mild deleterious phenotype we observe in pregnancy with chronic CRHR2 blockade. However, they do challenge our findings with total CRHR blockade in non-pregnant mice. In our experiments total CRHR blockade outside of pregnancy using α-helical CRF_{9-41} had no effects on glucose clearance, plasma insulin or insulin sensitivity suggesting that the effects seen with CRHR2 blockade are specific to pregnancy. Based on the previous studies it may have been expected that non-selective blockade of CRH receptors in non-pregnant animals would result in a similar phenotype of glucose impairment to that observed in pregnancy. However, there are obvious differences between studies, with Li et al using male rats, acutely administering a receptor antagonist intravenously compared to our chronic administration of a non-selective receptor antagonist to female mice. Furthermore, despite Astressin 2B and α-helical CRF_{9-41} having near identical EC50 values for mouse CRHR2β (1.3 nM and 1.1 nM respectively), the EC50 of α-helical CRF_{9-41} for CRHR1 is approximately 20x higher (19 nM) compared to type 2 CRHRs (Rivier and Rivier, 2014). Therefore, although α-helical CRF_{9-41} is capable of antagonising both subtypes of CRHR, it appears to have greater potency at antagonising CRHR2. It is therefore plausible that any type 1 CRHRs not totally blocked with the non-selective antagonist in non-pregnant mice, may have provided compensatory signalling to counterbalance any effects on glucose tolerance with α-helical CRF_{9-41}. However, this explanation is under the assumptions that the β-isoform of CRHR2 on β-cells is directly responsible for the phenotype we see in our pregnancy studies. Other reports have challenged the deleterious effects reported for CRHR2 antagonism on glucose tolerance as administration of Astressin 2B *in vivo* to wildtype mice showed improved glucose tolerance (A. Chen et al., 2006). Reports have also suggested that Astressin 2B may have weak partial agonist activity (Tasma et al., 2020) which further complicates any interpretations that can be made with these studies and our observations. Nevertheless, the phenotype we observed with blocking endogenous CRHR2 signalling (selectively and non-selectively) strongly suggest that this receptor may be specifically exploited during pregnancy to enhance β-cell function.

β-cell proliferation and hypertrophy are also cellular mechanisms that facilitate the expansion of the functional β-cell pool during rodent pregnancy. Several placental hormones involved in β-cell adaptations have demonstrated the ability to simultaneously regulate both the expansion and functional compensatory mechanisms. As discussed previously, exposure of pancreatic islets to lactogenic hormones increases the rate of β-cell proliferation (Brelje et al., 1993; Sorenson and Brelje, 1997; Vasavada et al., 2000) and indeed, reduced β-cell proliferation and failure to expand β-cell mass is exhibited with targeted deletion of their cognate receptor (PRLR) (Banerjee et al.,

2016b). More recently, a classical neuroendocrine hormone, kisspeptin, has emerged as a placental derived hormone involved in the mitogenic regulation of the β-cell responses to pregnancy. Chronic exposure of mice to Kisspeptin-234 (kisspeptin receptor antagonist) or more specifically, targeted deletion of the kisspeptin receptor (GPR54) in β-cells in pregnancy, causes a reduction in β-cell proliferation and contributes to the impaired glucose homeostasis of these mice during pregnancy (Bowe et al., 2019). In the current study, chronic blockade of total CRH receptors during pregnancy had no significant effects on β-cell size or proliferation, or on the overall β-cell mass. Although two separate studies have suggested CRHR1 activation can promote β-cell proliferation in either primary rat neonatal β-cells (Huising et al., 2010) or insulinoma cell lines (Schmid et al., 2011), these effects were mediated by CRH/CRHR1-selective ligands and CRHR1. These *in vitro* effects in cell lines may not completely reflect the physiology of islet cells *in vivo*, and based on our previous observation that islet CRHR1 expression is downregulated on day 16 of gestation, may suggest that the mitogenic effects of CRH/CRHR1 may not be so important or as influential as other signals whose levels surge during pregnancy (i.e. PL, PRL or kisspeptin). As no alterations in the rate of β-cell proliferation was detected following CRHR blockade during pregnancy, this provides further evidence that the impairment to glucose tolerance *in vivo* during pregnancy is due to an endogenous ligand, specifically targeting CRHR2, enhancing β-cell insulin secretion. It is intriguing that the placental ligand mediating these effects (i.e. UCN2) doesn't exert the dual effects on β-cell function and expansion as most ligands involved in the islet adaptation to pregnancy have demonstrated. Instead the mechanism appears to be limited to enhancing β-cell secretory responses. Often, placental signals regulating islet adaptations are studied in isolation, however all of the maternal β-cell adaptations may not occur independently but instead could be acting in concert, feeding into common signalling pathways. The selective endogenous ligand for CRHR2 could therefore represent a placental signal which functions to potentiate the effects of other placental ligands on β-cell insulin release. This concept has been demonstrated with other members of the CRH family for example with CRH and vasopressin. Both CRH and vasopressin can independently enhance GSIS however, vasopressin has been shown to potentiate CRH-induced insulin release from mouse pancreatic β-cells (O'Carroll et al., 2008). Whether the mechanistic difference observed with β-cell CRHR2 signalling/UCN2 in pregnancy is because it is signalling in synergy with another signalling mechanism is yet to be determined. Still, the physiological significance of these differences in modes of action of placental factors is uncertain however, there may be therapeutic advantages in the ability of UCN2 to enhance glucose-induced insulin secretion without targeting the clinical challenges of manipulating β-cell proliferation.

Varying degrees of maternal glycaemic control may be evident throughout pregnancy. This can range from normoglycaemia in the case of sufficient β-cell adaptive responses, to mild/moderate dysglycaemia or even severe as seen in gestational diabetes. The pharmacological blockade of

CRHR2 signalling during pregnancy appears to reveal a mild glucose intolerance, a notably transient effect in comparison to the more profound defect in glucose tolerance displayed by mutant PRLR mice (Huang et al., 2009). Given the importance of maintaining appropriate maternal glycaemic control during pregnancy, the mild phenotype displayed may reflect complementary signalling pathways compensating for the lack of CRHR2 signalling. The multiple hormones involved in regulating the islet adaptation to pregnancy provide critical refinement and possibly redundancy to ensure sufficient adaptation to prevent major disruptions to glucose homeostasis. As a result, blocking any one signal may produce a relatively mild phenotype but this does not necessarily mean the signal is unimportant. Another factor to consider is whether the dosing of the selective CRHR2 antagonist was sufficient to achieve maximal inhibition of CRHR2 on pancreatic β-cells throughout its chronic administration. Our estimated steady state concentration and the reported EC50 of the antagonist for CRHR2 (0.6 – 2.2 nM) (Rivier and Rivier, 2014) would suggest maximal blockade. Even if the antagonist was not 100% efficient (resulting in a mild phenotype), the impairment to glucose tolerance displayed during pregnancy with CRHR2 blockade would suggest that even minor inhibition of this signal could have a negative influence on maternal β-cell function and thus glycaemic control.

Overall, the above evidence suggests a novel role for CRHR2 signalling involved in β-cell adaptive responses in murine pregnancy to maintain maternal glucose homeostasis. Consistent with our observations thus far, endogenous placental UCN2 is the most likely signal mediating this adaptation via enhancing β-cell insulin secretory responses to elevated plasma glucose, especially in the prevailing insulin resistant environment. Unlike other identified placental signals, the effects of UCN2 appear to be confined to amplifying glucose-induced insulin secretion without concomitant alterations in the β-cell mass. Instead of displaying an overt gestational diabetic phenotype, the impairment to glucose tolerance was much milder when the endogenous CRHR2 agonist was blocked. This may suggest that UCN2 may act in concert with other placental signals to fine-tune the compensatory β-cell adaptations to maternal insulin resistance during pregnancy. Whether the beneficial insulinotropic effects of UCN2 could be exploited in other models of impaired glucose homeostasis/insulin resistance will be explored in the following chapter.

Chapter 5

Chapter 5 Effects of exogeneous UCN2 in an animal model of impaired glucose homeostasis

Chapter snapshot

Selectively blocking CRHR2 during mouse pregnancy caused an impairment to glucose tolerance suggesting the rise in endogenous UCN2 measured on PD.16 in mouse pregnancy has beneficial effects on maintaining maternal glucose tolerance.

No alterations to glucose or insulin tolerance were observed with CRHR blockade outside of pregnancy, possibly because endogenous UCN2 levels are low and glucose tolerance is controlled adequately.

Obese ob/ob mice display similar physiological features to that of rodent pregnancy (i.e. insulin resistance, hyperglycaemia and increased demand for insulin).

Therefore, the aim of this chapter was to investigate whether we could replicate the beneficial effects of endogenous UCN2 seen during pregnancy by chronically administering UCN2 to obese ob/ob mice as a model of impaired glucose homeostasis.

5.1 Introduction

The results discussed in the previous chapter (chapter 4) demonstrate that endogenous UCN2 plays an important role in mediating the islet adaptation to pregnancy. Signals involved in the β-cell adaptations to pregnancy may represent putative targets for developing novel therapeutic strategies. Other signals that have been identified to play a role in the islet adaptation and possibly GDM, are currently being investigated for their therapeutic potential. Downstream β-cell PRLR serotonergic signalling (stimulated by pregnancy lactogens) is well recognised as a fundamental mechanism in mediating β-cell adaptations in response to pregnancy (Ohara-Imaizumi et al., 2013). Intra-islet serotonin (5-HT) signalling via serotonin receptors (i.e. 5-HTR3) has been

shown to increase the glucose responsiveness of β-cells and thereby increase the overall islet GSIS to compensate the maternal insulin demand (Oh et al., 2016; Ohara-Imaizumi et al., 2013). Strategies that target the serotonergic system, for example, drugs that agonise the 5-HTR3 receptor, which has been illustrated *in vitro* to improve β-cell GSIS, may therefore offer a new therapeutic target for diabetes (Moyce and Dolinsky, 2018; Oh et al., 2016; Ohara-Imaizumi et al., 2013).

Another promising molecular target identified to be involved in the islet adaptive mechanisms in pregnancy is placental kisspeptin. Several studies have documented the dramatic elevation in plasma kisspeptin levels throughout human pregnancy (Dhillo et al., 2006; Horikoshi et al., 2003a). In the non-pregnant state, plasma kisspeptin levels are below 2 pmol/l. By the first trimester, kisspeptin levels are approximately 400-fold higher and by the third trimester, approximately 1,000-fold higher compared to non-pregnant levels. By 15 days postpartum, circulating kisspeptin returns to pre-gestational levels (Dhillo et al., 2006). Accumulating evidence has supported a physiological role for the higher circulating kisspeptin levels during normal pregnancy to positively influence maternal glucose homeostasis by enhancing insulin secretion and supporting β-cell mass expansion (Bowe et al., 2019). In keeping with this hypothesis, pregnant women with GDM have lower circulating levels of kisspeptin compared to pregnant women without GDM (Bowe et al., 2019). Therefore, kisspeptin represents an attractive diagnostic marker for GDM or even a potential anti-diabetic therapy. Accordingly, a recent study has demonstrated that administration of kisspeptin (1 nmol $kg^{-1}h^{-1}$) to 15 healthy men enhances insulin secretion following an intravenous glucose load, providing the first demonstrations of the potential beneficial metabolic therapeutic application of kisspeptin therapy *in vivo* in humans (Izzi-Engbeaya et al., 2018). Phase 1 clinical trials looking at the influence of kisspeptin (112-121) in regulating blood sugar and insulin levels are also currently ongoing (https://clinicaltrials.gov/ct2/show/NCT02953834). Both therapeutic targets described (i.e. 5-HTR3 agonists or kisspeptin) provide convincing evidence that molecular targets or signalling mechanisms involved in islet adaptive responses to pregnancy can offer attractive therapeutic strategies for diabetes. Therefore, endogenous UCN2 may also represents a molecular target that may be utilised in either the screening/diagnosis or treatment of GDM.

However, UCN2 is also present outside of pregnancy and has previously been shown to play multiple roles in the response to various physiological states. Due to its wide-ranging expression, UCN2 has been shown to play diverse biological roles in cardiovascular physiology (where it has demonstrated cardioprotective effects in heart failure) (Adão et al., 2015), modulation of inflammatory responses (mediating pro-inflammatory pathways for example in myometrial and intestinal cells (Novembri et al., 2015; Paschos et al., 2009; Voltolini et al., 2015) as well as in the local modulation of adrenal function (inducing catecholamine synthesis and release)

(Tsatsanis et al., 2007). Consequently, UCN2 is emerging as a clinically relevant molecule in the treatment and/or management of several conditions including, heart failure, upper gastrointestinal inflammatory diseases, anxiety and stress related behaviours, and more recently, in metabolic disorders (Bagosi et al., 2018; Gao et al., 2019; Gravanis and Margioris, 2005; Rademaker Miriam T. et al., 2011; Tsatsanis et al., 2007). From the results in the previous chapter it is unclear whether the beneficial effects of UCN2 on islet function are pregnancy-specific, or whether UCN2 is also involved in islet responses to other physiological states of insulin resistance.

The generation and study of UCN2-deficient transgenic mice has revealed a metabolic phenotype, although a limited number of studies have generated a considerable degree of contradictory data. Initial observations demonstrated that UCN2-deficient mice display significantly improved glucose tolerance and insulin sensitivity. This was attributable to significantly increased whole body glucose utilization (i.e. peripheral insulin sensitivity) as fasting and glucose-induced elevated insulin levels were similar between UCN2-null mice and WT littermates, suggesting that enhanced glucose tolerance in mutant mice was not due to increased insulin secretion. (A. Chen et al., 2006). Furthermore, hyperinsulineamic-euglycaemic glucose clamp studies also demonstrated that higher glucose infusion rates were required to maintain euglycaemia in UCN2-null mice compared to WT littermates. This was shown to be a consequence of significantly increased whole body glucose uptake, glycolysis and insulin-mediated suppression of hepatic glucose production as well as increased deoxy-[2-^{3}H] glucose uptake in the skeletal muscle of UCN2-null mice compared to WT littermates (A. Chen et al., 2006). By studying the effects of UCN2 in skeletal muscle cells it was determined that UCN2 inhibits insulin-induced phosphorylation of Akt as well as ERK1/2, consistent with the suggestion that UCN2 inhibits insulin signalling pathways. Therefore, the authors suggested that suppression of the UCN2/CRHR2 pathway may have therapeutic potential in insulin resistant T2DM (A. Chen et al., 2006).

Contrary to this, two individual studies have supported a favourable effect on glucose homeostasis in mice, through either overexpressing UCN2 (through gene transfer) or treatment with modified UCN2 peptide (Borg et al., 2019; Gao et al., 2019). Intravenous delivery of a vector (adeno-associated virus type 8 (AVV8)) encoding a transgene for *Ucn2* was administered in two separate animal models of insulin-deficiency, the Akita mouse or the Streptozotocin model (Gao et al., 2019). The Akita mouse represents a monogenic, non-obese model of insulin deficiency caused by a spontaneous mutation in the insulin 2 gene whereas the Steptozotocin mouse model is a chemically induced model of T1DM where a high percentage of endogenous β-cells are selectively destroyed (King, 2012). UCN2 gene transfer proved to be efficacious in improving glycaemic control in both mouse models even after several months following single intravenous injection. Assessment of glucose stimulated insulin release in 5-month-old Akita mice (2 months

after vector delivery), revealed AVV8-*Ucn2* treated mice showed increased plasma insulin (3-fold higher) than those seen in AAV8-null mice 120 min after glucose administration. Similarly, in Streptozotocin mice UCN2 gene transfer was associated with reduced area under the curve (AUC) in glucose tolerance tests (Gao et al., 2019). Furthermore, in hyperinsulineamic-euglycaemic clamp studies, the glucose infusion rate was 2.8-fold higher in Akita AAV8-*Ucn2* mice compared to AAV8-null mice, indicating increased insulin sensitivity which was subsequently shown to be particularly enhanced in skeletal muscle (Gao et al., 2019). Thus, UCN2 gene transfer appeared to demonstrate combined effects of increasing both insulin release and sensitivity *in vivo*.

Gao and colleagues have also previously reported beneficial effects of UCN2 gene transfer in mouse models of insulin resistance, including high fat diet (HFD)-induced and in the leptin receptor deficient db/db mouse (Gao et al., 2016). These animal models are obese, hyperinsulineamic and hyperglycaemic reflecting T2DM which is commonly associated with insulin resistance (King, 2012). A single injection of AAV8-*Ucn2* normalised blood glucose levels and glucose disposal in these mice as evidenced by hyperinsulineamic-euglycaemic clamp studies showing reduced plasma insulin, increased glucose disposal rates and increased insulin sensitivity. The authors further demonstrated a potential mechanism by which UCN2 operates to increase insulin sensitivity by showing that GLUT-4 translocation to the plasma membrane was increased in skeletal myotubes following exposure to UCN2 peptide (200 nM) (Gao et al., 2016). Moreover, mice with CRHR2 deletion that were rendered insulin resistant by HFD showed no improvements in glucose disposal after UCN2 gene therapy, indicating that the insulin sensitizing effects promoted by UCN2 requires signalling via its cognate receptor (Gao et al., 2016).

Studies by Borg et al (2019) have similarly reported that chronic activation of CRHR2 with a modified PEGylated peptide analogue of human UCN2 injected subcutaneously daily for 14 days (0.3 mg/kg), improved glucose tolerance *in vivo* in HFD mice. These effects were mainly attributed to enhanced skeletal muscle insulin sensitivity as a result of increased GLUT-4 trafficking rather than improved islet function (Borg et al., 2019). Contrary to the study by Chen and colleagues mentioned above (A. Chen et al., 2006), the latter studies using UCN2 gene transfer and modified UCN2 peptide (Borg et al., 2019; Gao et al., 2019, 2016) support UCN2/CRHR2 activation as a potential therapeutic approach for either T1DM or T2DM. Evidently, there still remains uncertainty with regards to the peripheral effects of UCN2 on modulating glucose homeostasis including the mechanism (s) (i.e. promoting insulin availability and/or effectiveness) by which the peptide mediates its effects.

Current therapeutic approaches to GDM are similar to those of T2DM, including diet and lifestyle interventions and possibly combining insulin sensitizers and insulin secretagogues, reflecting the

similarities in aetiology of both conditions. Defective insulin secretion and insulin resistance are key pathological mechanisms in the progression of GDM and T2DM, compounded by other risk factors (Pedersen, 2013), so any putative therapy identified to be effective in pregnancy may also be effective in models of T2DM. However, whilst the aetiology of GDM and T2DM are similar, the underlying signals and hormonal milieu are very different. Though the beneficial effects of UCN2 on islet function in pregnancy may potentially translate into a non-pregnant model of insulin resistance and glucose intolerance, this is far from certain. It is thus essential to investigate the effects of UCN2 in a model of T2DM to determine whether the beneficial effects on islet function are pregnancy-specific or more generally applicable.

The morbidly obese ob/ob mouse model is commonly used as a model of obesity, insulin resistance and mild type 2 diabetes. Hence, these mice are widely used to study new therapies to improve insulin resistance or treatments to improve β-cell function (King, 2012). The phenotype of ob/ob mice is attributable to a single gene mutation resulting in the lack of the functional polypeptide hormone, leptin (King, 2012; Zhang et al., 1994). Leptin is a product of the *ob* gene and is predominantly synthesised and secreted by adipocytes (Huang and Li, 2000). Leptin levels reflect adipocyte size and body-fat mass. It has a pivotal role in signalling satiety to limit food intake as well as regulating whole-body energy expenditure via signalling in the brain (Zhou and Rui, 2013). Defective or lack of leptin signalling induces hyperphagia and subsequent obesity and is associated with extreme insulin resistance and glucose intolerance, along with a myriad of other endocrine/metabolic abnormalities similar to those seen in obese humans with T2DM (Wang et al., 2014).

In ob/ob mice, considerable weight gain is noticeable at 2 weeks of age and by 4 weeks, hyperglycaemia and hyperinsulinemia are apparent (Lindström, 2010). These mice also display a dramatic increase in pancreatic islet volume, containing a high proportion of insulin-producing β-cells (Bock et al., 2003). Abnormal glucose and insulin tolerance are exhibited by ob/ob mice, both worsening with age. Although there are some abnormalities in insulin release (Lavine et al., 1977; Lindström, 2010), islets maintain insulin secretion and the lack of complete β-cell failure in this model means diabetes is not particularly severe. Nevertheless, persistent hyperglycaemia in ob/ob mice is still indicative of insufficient β-cell function, despite hyperinsulinemia. Pancreatic islets from many ob/ob strains have a high capacity to secrete insulin and they respond adequately to most stimulators and inhibitors of insulin release (Beloff-Chain and Hawthorn, 1976; Hellman et al., 1974; Lindström, 2010). In fact, sensitivity to insulin releasing hormones, including incretin therapies, have been demonstrated in ob/ob mice. Thus, GLP-1 or GIP analogues or dipeptidyl peptidase-4 (DPP-4; enzyme which degrades incretins) inhibitors, have been reported to stimulate β-cell proliferation and glucose-induced insulin release, improving β-cell function and glycaemic control in ob/ob mice (Lindström, 2010; Moritoh et

al., 2008; O'Harte et al., 2000; Rolin et al., 2002; Young et al., 1999). These studies support the idea that even under prolonged functional stress of high insulin demand, ob/ob β-cells can still respond to insulin secretagogues and may therefore, also be influenced by mediators of β-cell adaptations associated with pregnancy.

Unsurprisingly, the ob/ob mouse displays similar physiological features to that of rodent pregnancy; 1. Progressive insulin resistance, 2. Hyperglycaemia, 3. Increased demand for insulin secretion and 4. Extensive hyperplastic and hypertrophic β-cell mass. However, these changes occur in response to the lack of leptin signalling, without the characteristic changes in the maternal hormonal milieu. The mechanisms driving the functional and morphological changes in either physiological states are clearly quite distinct, with hyperglycaemia (indirectly or directly) probably stimulating adaptations in the obese ob/ob mouse, whereas placental signals have a more influential role in pregnancy. The ob/ob mouse is therefore a good alternative model of impaired glucose homeostasis which can be used to investigate whether UCN2 may be able to improve glucose homeostasis by mimicking the effects anticipated during pregnancy.

5.2 Materials and Methods

5.2.1 Animals

All animal procedures were conducted under approval by King's College London Animal Welfare and Ethical Review Board and were undertaken in accordance with the United Kingdom Home Office Animals (Scientific Procedures) Act 1986, complying with the appropriate project and personal licence. Animals were housed under controlled conditions (12-hour light (0700-1900) /dark cycle, temperature 22 ± 2^{o}C) and provided with standard chow diet and water *ad libitum*.

All experimental animals used were either male C57BL/6, ob/ob (Envigo Laboratories, Bicester, UK) or male CD1 mice (Charles River Laboratories, Harlow, UK), aged between 8 – 16 weeks. The studies described below, utilised all mouse strains to investigate the acute effects of exogenous UCN2 i.p. administration on glucose homeostasis. Male C57BL/6 and ob/ob mice were both utilised to also study the effects of chronic administration of UCN2 via subcutaneously implanted osmotic minipumps on glucose and insulin tolerance.

5.2.2 Acute i.p. administration of UCN2

Male CD1, C57BL/6 or ob/ob mice were administered UCN2 (0.1 mg/kg) (Sigma, UK) or saline (equivalent volume) via i.p. injection. For mice subjected to glucose tolerance tests, fasting from 0900 for 6 hours was carried out and animals subsequently weighed. Mice were administered with UCN2 20 minutes prior to i.p. glucose challenge (2 g/kg) (Sigma, UK) as detailed in Figure 5-1. Baseline blood glucose and blood sampling was carried out after UCN2 administration and prior to glucose load. Blood glucose levels were determined (sampled from a small tail prick) at set time intervals over a 2.5-hour period using Accu-Chek glucose meter and strips (Roche Diagnostics, UK) or NOVA Statstrip® Xpress meter and appropriate test strips (Data Sciences International, USA) (see section 2.6.1 for detailed method). During the course of the GTT, additional blood sampling was carried out at 30 mins post glucose administration for the subsequent measurement of plasma insulin (described in section 2.6.1.1) using a commercial mouse insulin ELISA (10-1247-01, Mercodia, Sweden) following the manufacturer's instructions.

In a separate experiment, CD1, C57BL/6 and ob/ob mice were subjected to UCN2 peptide (0.1 mg/kg)/saline administration and the consequence on circulating blood glucose levels were examined independent of glucose load. Baseline blood glucose measurements were determined via small tail vein prick prior to i.p. injection of peptide/saline as per Figure 5-2. Subsequent blood glucose levels were measured via small tail vein prick over a 1-hour time course, using

Accu-Chek glucose meter and strips (Roche Diagnostics, UK) or NOVA Statstrip® Xpress meter and appropriate test strips (Data Sciences International, USA).

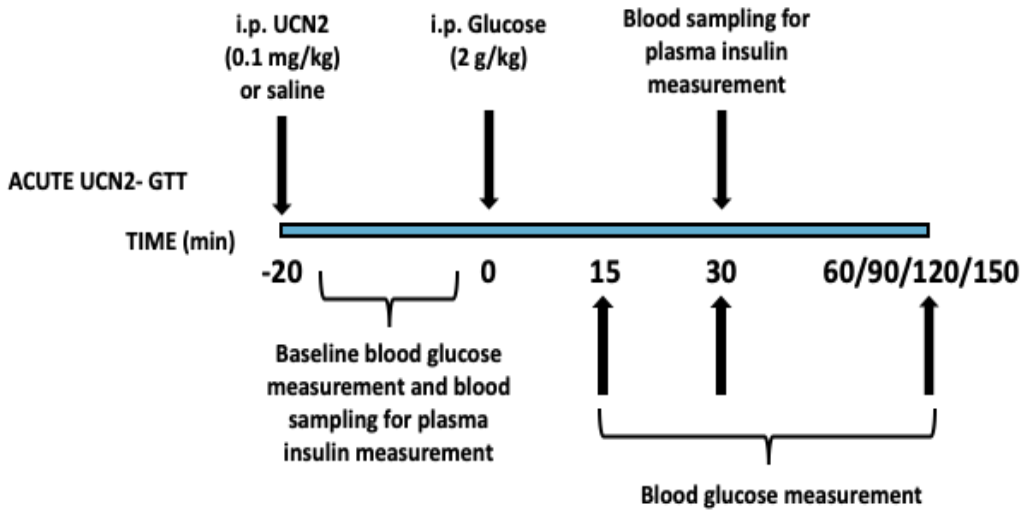

Figure 5-1 Experimental timeline for acute i.p. UCN2 and glucose tolerance testing in male C57BL/6 and ob/ob mice.

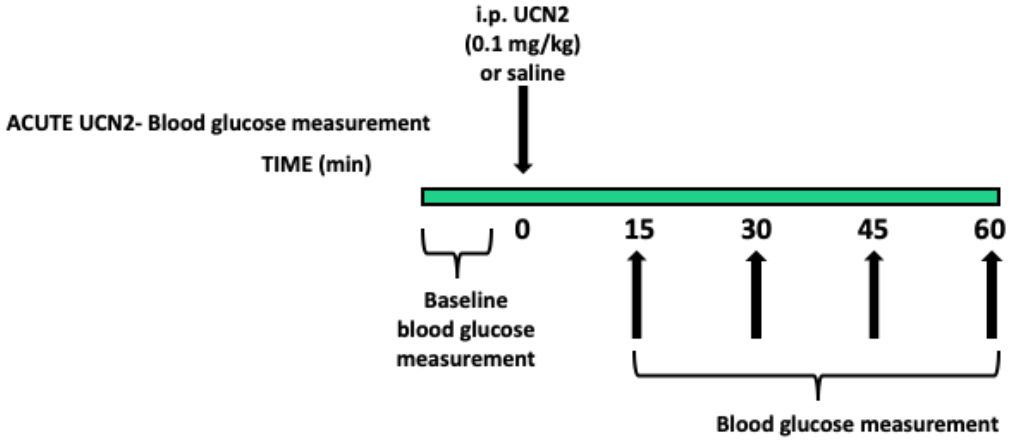

Figure 5-2 Experimental timeline for acute i.p. UCN2 and measurement of blood glucose in male CD1, C57BL/6 and ob/ob mice.

5.2.3 Chronic administration of UCN2 via osmotic minipumps (OMPs)

In attempt to mimic the chronic physiological circulatory levels of UCN2 measured in mouse pregnancy (Chapter 4, Figure 4-2D), either lean C57BL/6 or obese ob/ob mice were implanted subcutaneously with OMPs (ALZET®, Model 1002, Charles River, UK) (as described in section 2.5.1) containing saline for control mice or two separate doses of UCN2 (Dose 1: 83.04 µg/ml ~ 5 pmol/h; Dose 2: 415.2 µg/ml ~ 25 pmol/ h) (Generon, UK). Mice subsequently underwent fasted i.p. GTT (2 g/kg), ITT (0.75 IU/kg) and a final GTT on day 7, day 10 and day 14 post OMP implant respectively (as per experimental timeline in Figure 5-3). Mice were also weighed daily in the morning for the duration of the study.

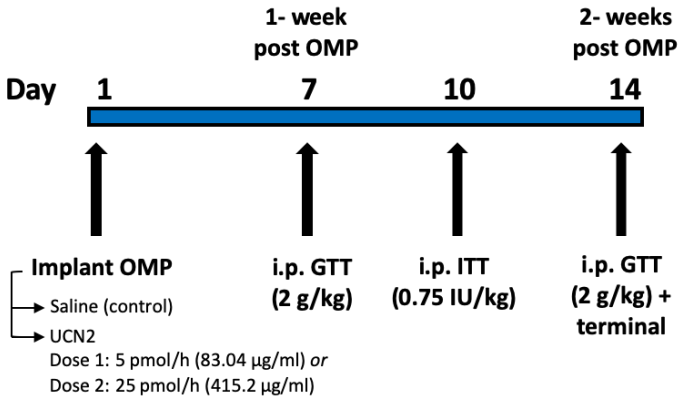

Figure 5-3 Experimental timeline for OMP studies

5.2.3.1 Calculation for dosing of chronic UCN2

An approximate calculation was performed as per the principles and equations detailed in Chapter 4 (4.2.4.1) to determine the rate of UCN2 infusion and thus the concentration of the peptide required to be loaded into the osmotic minipump such that a concentration of UCN2 similar to the plasma levels measured in mouse pregnancy (i.e. ~200 pmol/l- Chapter 4) were achieved at steady state. The biological half-life of UCN2 used as a reference was 15 minutes (reported in sheep) (Patel et al., 2012).

5.3 Results

5.3.1 Acute effects of UCN2 on glucose tolerance

Administration of UCN2 via single intraperitoneal (i.p.) injection to both healthy lean C57BL/6 mice and obese ob/ob mice allowed for the study of the acute effects of the peptide on glucose clearance. All mice responded to i.p. administration of glucose, elevating blood glucose levels within 15 mins as shown in Figure 5-4. As expected, ob/ob animals exhibited an overall worse glucose tolerance compared to lean saline treated animals as illustrated by both absolute blood glucose values and total glucose AUC in Figure 5-4A and Figure 5-4B respectively. UCN2 treatment caused a significant worsening of glucose tolerance in both lean and ob/ob mice compared to their respective saline treated counterparts, with significantly higher blood glucose levels observed from as early as 15 mins in lean mice (continued for the entire 150 min duration) and from 120 mins in ob/ob mice (Figure 5-4A). Again, as expected ob/ob mice were hyperinsulinemic, with significantly higher fasting plasma insulin levels compared to lean mice (ob/ob (saline) vs lean (saline): 0 min; 1517.03 ± 20.33 vs 166.86 ± 18.15 pM) (Figure 5-4C). Glucose induced plasma insulin release at 30 min was ~ 34% lower in lean UCN2-treated mice compared to saline controls and was also significantly reduced in ob/ob UCN2-treated mice compared to saline control (Lean (saline) vs lean (UNC2): 30 min; 197.05 ± 37.25 vs 129.82 ± 18.29 pM; ob/ob (saline) vs ob/ob (UCN2): 30 min; 1474.65 ± 34.71 vs 1250.89 ± 60.11, ***p<0.001) (Figure 5-4C).

5.3.2 Acute effects of UCN2 on circulating blood glucose levels

To further investigate the acute effects of UCN2 *in vivo*, the consequences of peptide treatment on circulating blood glucose levels independent of glucose challenge were examined. Male CD1, C57BL/6 and ob/ob mice all displayed a similar elevation in blood glucose concentrations in response to a single i.p. administration of UCN2 (Figure 5-5). Whereas control (saline) CD1 mice maintained blood glucose levels at around 8 mM throughout the testing period, mice treated with UCN2 showed a significant elevation in blood glucose from 45 min (~11 mM), further increasing at 60 min (~12 mM) (Figure 5-5A). Area under the curve quantification for total blood glucose levels, also confirms the significant increase in blood glucose with UCN2 treatment (AUC saline vs UCN2; 496 ± 31.0 vs 609 ± 31.8, *p<0.05) as shown in Figure 5-5B. C57BL/6 mice appeared to be more sensitive to the effects of UCN2, with significantly higher blood glucose levels from 30 mins which remained elevated for a further 30 mins in comparison to control mice (AUC saline vs UCN2; 686.1 ± 22.0 vs 960.6 ± 91.4, *p<0.05) (Figure 5-5C & D). Again, a similar response was exhibited by ob/ob mice, with overall higher glucose levels in animals administered UCN2 (AUC saline vs UCN2; 1206 ± 59.0 vs 1404 ± 67.0, p=0.0503) (Figure 5-5E & F).

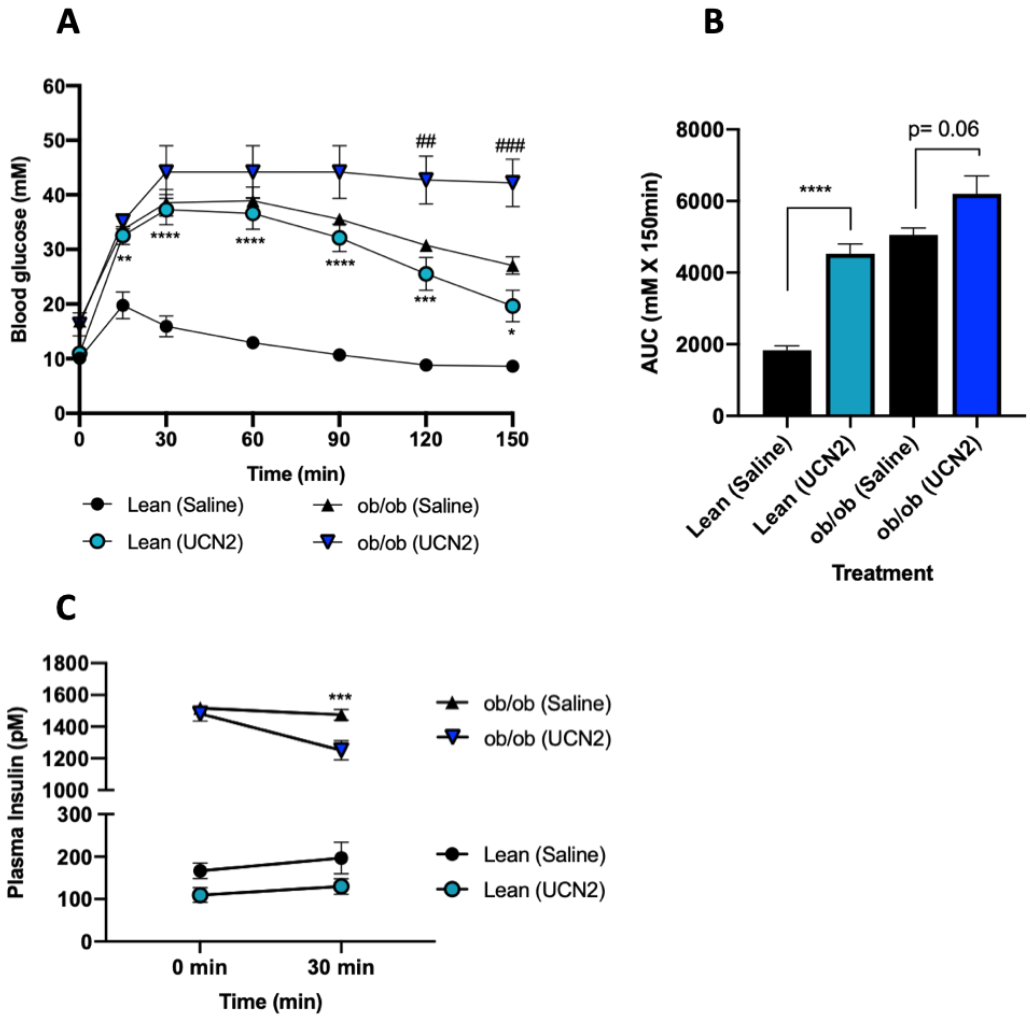

Figure 5-4 Acute effects of UCN2 on glucose tolerance in male C57BL/6 and ob/ob mice. C57BL/6 or ob/ob mice were either single i.p. administered saline (black circles and black triangles respectively) or UCN2 (teal circles and blue triangles respectively) 20 minutes prior to fasted i.p. GTT (A). Representative glucose AUC for entire test duration (0-150 min) for saline treated animals (black bars) and UCN2 treated mice (teal and blue bars) are displayed (B). GTT fasted and 30 min post glucose administration plasma insulin for saline and UCN2-treated C57BL/6 mice (black circles and teal circles respectively) as well as saline and UCN2-treated ob/ob mice (black triangles and blue triangles respectively) as shown in (C). Data are presented as mean ±SEM, n = 5-6 mice/group, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (lean saline vs lean UCN2), #p<0.05, ##p<0.001 (ob/ob saline vs ob/ob UCN2), two-way RM-ANOVA or one-way ANOVA followed by Tukey's / Sidak's multiple comparisons test.

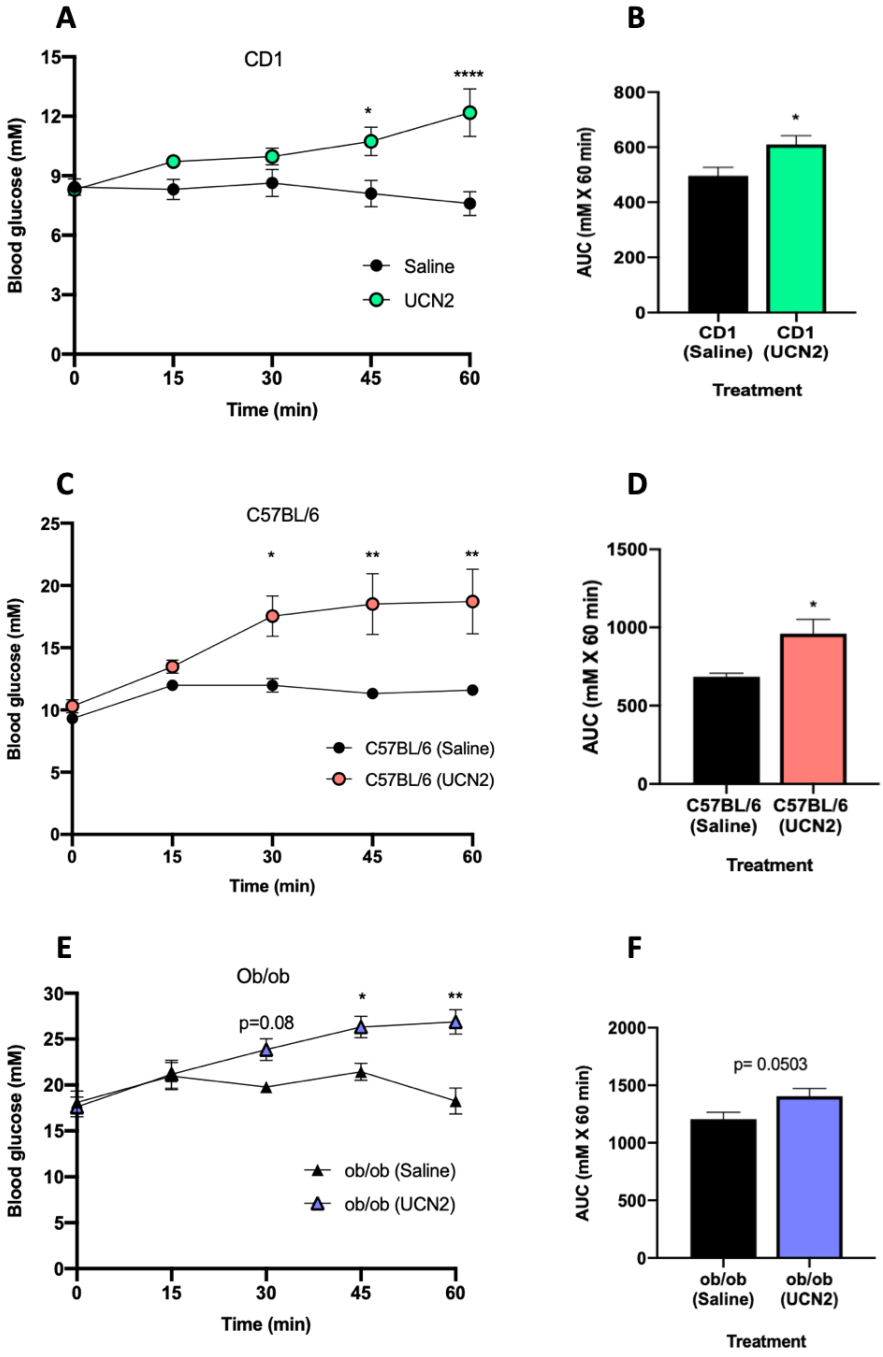

Figure 5-5 Acute effects of UCN2 on circulating blood glucose levels. Male CD1(A), C57BL/6 (C) and ob/ob (E) mice were single i.p. administered saline (black icons/ bars) or UCN2 (lime, peach or purple icons/ bars) and blood glucose was measured via small tail prick at 15 min time intervals over 1- h. Representative AUC for total blood glucose levels are shown in (B), (D) and (F) for CD1, C57BL/6 and ob/ob mice respectively. Data are presented as mean ±SEM, n = 6 mice/group, *p<0.05, **p<0.01, ****p<0.0001 (saline vs UCN2), two-way RM-ANOVA followed by Sidak's multiple comparisons test. AUC data analysed by two-tailed t-test.

5.3.3 Effects of chronic UCN2 administration on glucose homeostasis in lean C57BL/6 mice – Dose 1: 5 pmol/h

To mimic the chronic physiological circulatory levels of UCN2 as seen in mouse pregnancy (Chapter 4 Figure 4-2D), C57BL/6 mice were subcutaneously implanted with OMPs chronically infusing UCN2 (Figure 5-6). Baseline parameters, such as body weight and glucose tolerance were comparable between both randomly allocated treatment groups of mice, prior to OMP implantation (Figure 5-6A & B). Following implantation of OMPs, no differences were observed in the visual appearance of saline treated or UCN2 treated mice or in the rate of weight gain over the two-week study period (Figure 5-6C & D). Equally, glucose tolerance was unchanged between control and UCN2 treated animals, both at 1 week and 2 weeks of UCN2 infusion as shown in (Figure 5-6E & F). In fact, by the end of the study both control and UCN2 treated mice exhibited comparable glucose tolerance to baseline parameters as indicated by the area under the curve quantification for total blood glucose levels for each time point (Figure 5-6G). Mice treated with UCN2 revealed a transient impairment in insulin sensitivity (10 days post OMP) displayed at 15 min post insulin administration (UCN2 vs saline: 15 min, 7.13 ± 0.85 vs 5.13 ± 0.34 mM, *$p<0.05$) (Figure 5-6H) but this was not sustained at further time points and overall insulin tolerance was analogous between saline and UCN2 treatments (AUC saline vs UCN2; 335.0 ± 11 vs 372.4 ± 21.1, $p=0.217$) (Figure 5-6H insert).

5.3.4 Effects of chronic UCN2 administration on glucose homeostasis in lean C57BL/6 mice – Dose 2: 25 pmol/h

In a following study employing a higher dose of UCN2 infusion, the chronic effects of the peptide on glucose homeostasis were again investigated in C57BL/6 mice (Figure 5-7). Control (saline) and UCN2 treated mice were matched for weight and overall baseline glucose tolerance as demonstrated by Figure 5-7A & B. Similarly to animals infused with the lower dose of UCN2 (Figure 5-6), no significant differences were observed in rate of weight gain or glucose tolerance over the two -week study period (Figure 5-7C-F). In contrast to the lower dose of UCN2, insulin tolerance transiently and significantly improved, specifically at 15 min post insulin administration in UCN2 treated animals as assessed 10 days post peptide infusion (Figure 5-7G). However, overall insulin tolerance was equivalent between control and UCN2 treated animals (Figure 5-7G insert).

Dose 1: 5 pmol/h

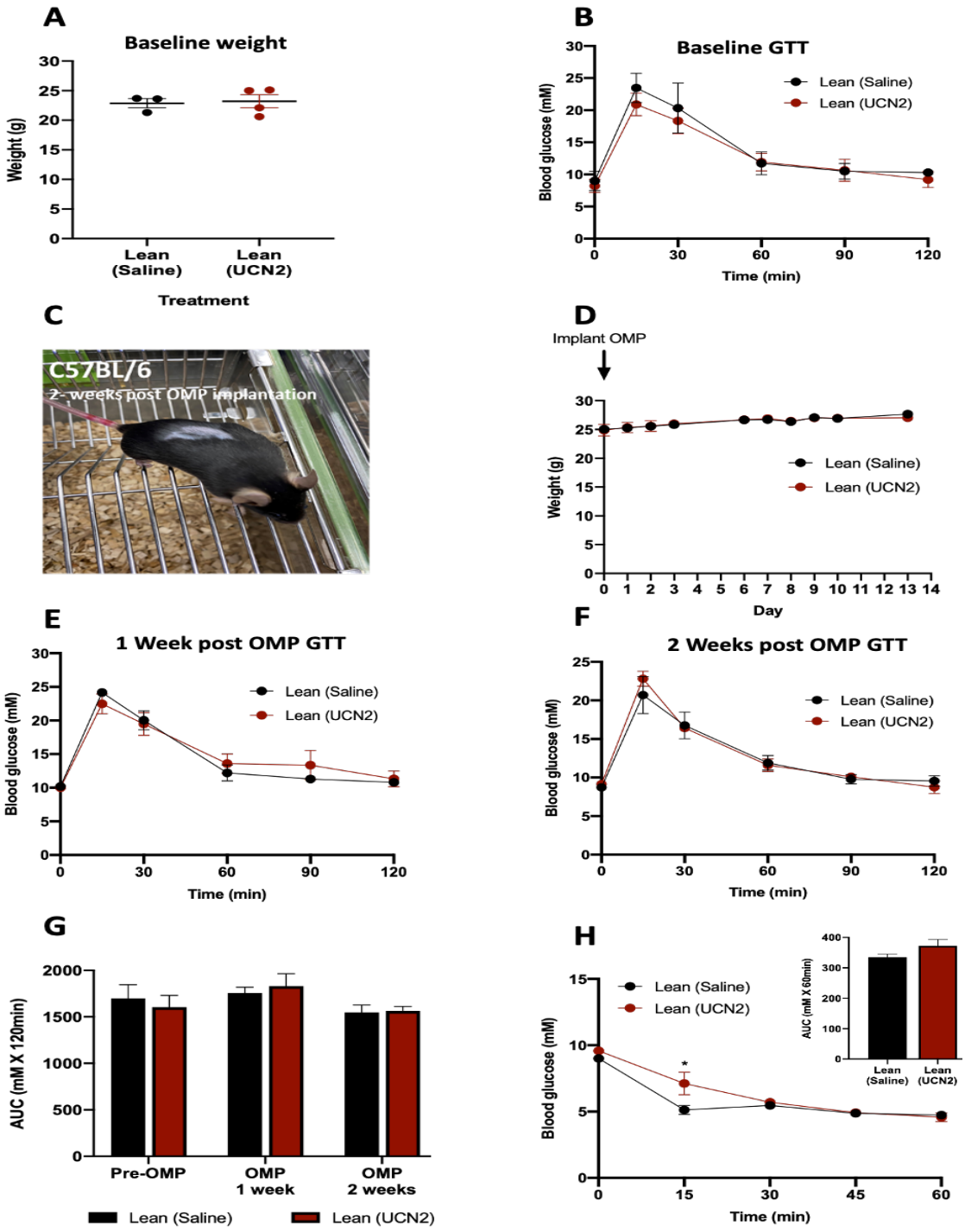

Figure 5-6 Chronic effects of UCN2 on glucose homeostasis in C57BL/6 mice. Dose 1: 5 pmol/h (Figure legend on following page)

Figure legend continued

Figure 5-6 Chronic effects of UCN2 on glucose homeostasis in C57BL/6 mice – Dose 1: 5 pmol/h. Male C57BL/6 mice were subcutaneously implanted with OMPs chronically infusing saline (black circles/bars) or UCN2 (red circles/bars). Baseline weight (A) and glucose tolerance (B) were recorded prior to OMP implantation. Image of mouse taken at the end of the study (C) and weight was monitored throughout the duration of the study (D). Fasted i.p. GTTs performed 1-week (E) and 2 weeks (F) post peptide infusion. Representative AUC for baseline (pre-OMP), 1-week and 2 -week GTT compared in (G) and fasted i.p. ITT (performed on day 10 post infusion) shown in (H) with respective AUC for entire test (0-60 min) in insert. Data are presented as mean ±SEM, n= 3-4 mice/group, **p*< 0.05 two-way RM-ANOVA/ two-way ANOVA followed by Sidak's / Tukey's multiple comparisons test.

Dose 2: 25 pmol/h

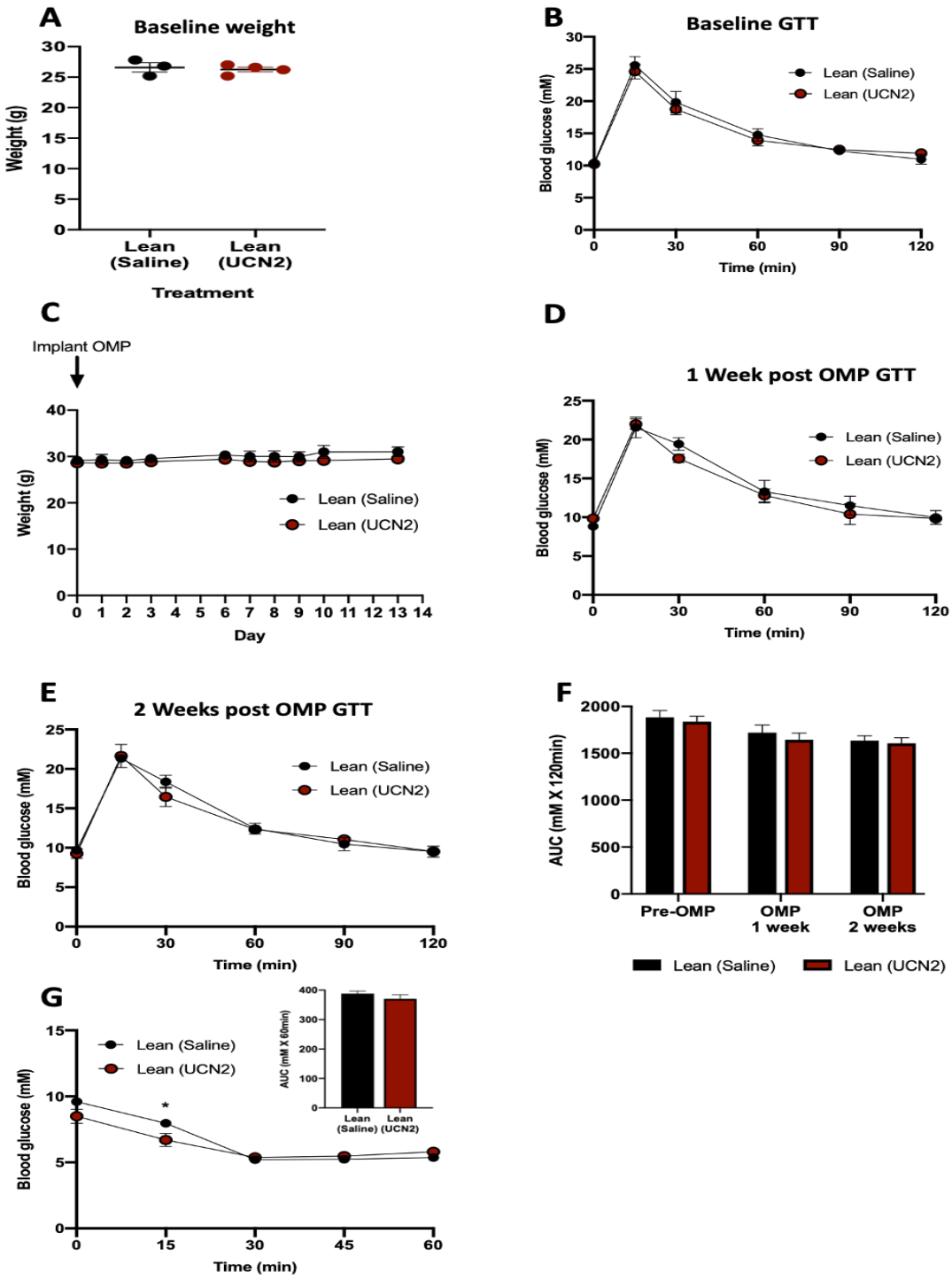

Figure 5-7 Chronic effects of UCN2 on glucose homeostasis in C57BL/6 mice. Dose 2: 25 pmol/h (Figure legend on following page)

Figure legend continued

Figure 5-7 Chronic effects of UCN2 on glucose homeostasis in C57BL/6 mice- Dose 2: 25 pmol/h. Male C57BL/6 mice were subcutaneously implanted with OMPs chronically infusing saline (black circles/bars) or UCN2 (red circles/bars). Baseline weight (A) and glucose tolerance (B) were recorded prior to OMP implantation. Weight was monitored throughout the duration of the study (C). Fasted i.p. GTTs performed 1-week (D) and 2- weeks (E) post peptide infusion. Representative AUC for baseline (pre-OMP), 1-week and 2-week GTT compared in (F) and fasted i.p. ITT (performed on day 10 post infusion) shown in (G) with respective AUC for entire test (0-60 min) in insert. Data are presented as mean ±SEM, n= 3-4 mice/group, *$p< 0.05$ two-way RM-ANOVA/ two-way ANOVA followed by Sidak's / Tukey's multiple comparisons test.

5.3.5 Effects of chronic UCN2 administration on glucose homeostasis in obese ob/ob mice – Dose 1: 5 pmol/h

Using a model of impaired glucose homeostasis, obese ob/ob mice were subcutaneously implanted with OMPs chronically infusing UCN2 to mimic the physiological circulatory levels of the peptide in mouse pregnancy measured in the previous chapter (Chapter 4, Figure 4-2D) (Figure 5-8). At baseline (pre-OMP implant) no significant differences were seen in either body weight or glucose tolerance between saline (control) and UCN2 treatment groups (Figure 5-8A & B). The ob/ob mice continued to rapidly gain weight following OMP implant however, the rate of weight gain was similar between both treatment groups (Figure 5-8D) and no visual or behavioural alterations were displayed by mice throughout the duration of the study (Figure 5-8C). After 1 week of peptide infusion, no significant differences in glucose tolerance between saline or UCN2 treated mice were observed as shown in Figure 5-8E. After 2- weeks, mice treated with UCN2 appeared to have a slower rate of glucose clearance compared to control mice, although this was not statistically significant (Figure 5-8F). Area under the curve quantification for total blood glucose levels at baseline, 1- and 2- weeks post OMP implant (Figure 5-8G) also indicated that this impairment was not statistically significant (AUC OMP 2 weeks: saline vs UCN2; 3912 ± 260 vs 4419 ± 133, $p=0.687$). Glucose tolerance was significantly worsened for UCN2-treated ob/ob mice by 2 weeks compared to their baseline, pre-OMP glucose profile (AUC Pre-OMP vs OMP 2 weeks ob/ob UCN2; 3067 ± 281 vs 4419 ± 133, $^{**}p<0.01$). Similarly, glucose tolerance was also significantly worsened for saline-treated ob/ob mice, evident at 1-week post OMP, compared to their baseline glucose profile, an effect which disappeared by 2- weeks (AUC Pre-OMP vs OMP 1 week ob/ob saline; 2984 ± 220 vs 4298 ± 348, $^{*}p<0.05$) (Figure 5-8G). As expected, ob/ob mice were insulin resistant as illustrated by their unresponsiveness to insulin administration and a lack of any reduction in blood glucose throughout an insulin tolerance test conducted 10 days after peptide infusion (Figure 5-8H).

5.3.6 Effects of chronic UCN2 administration on glucose homeostasis in obese ob/ob mice – Dose 2: 25 pmol/h

In a subsequent study employing a higher dose of UCN2 infusion, the chronic effects of the peptide on glucose homeostasis were again investigated in male ob/ob mice (Figure 5-9). Again, no significant differences were observed between saline or UCN2 treated animals in all parameters measured such as baseline body weight, baseline glucose tolerance or weight gain following OMP implant (Figure 5-9A-C). Metabolic testing throughout the 2 week study also revealed no significant differences in glucose clearance or insulin sensitivity although the metabolic profile of this cohort of animals was noticeably more variable than the previously studied cohort (Figure 5-9D-G).

Dose 1: 5 pmol/h

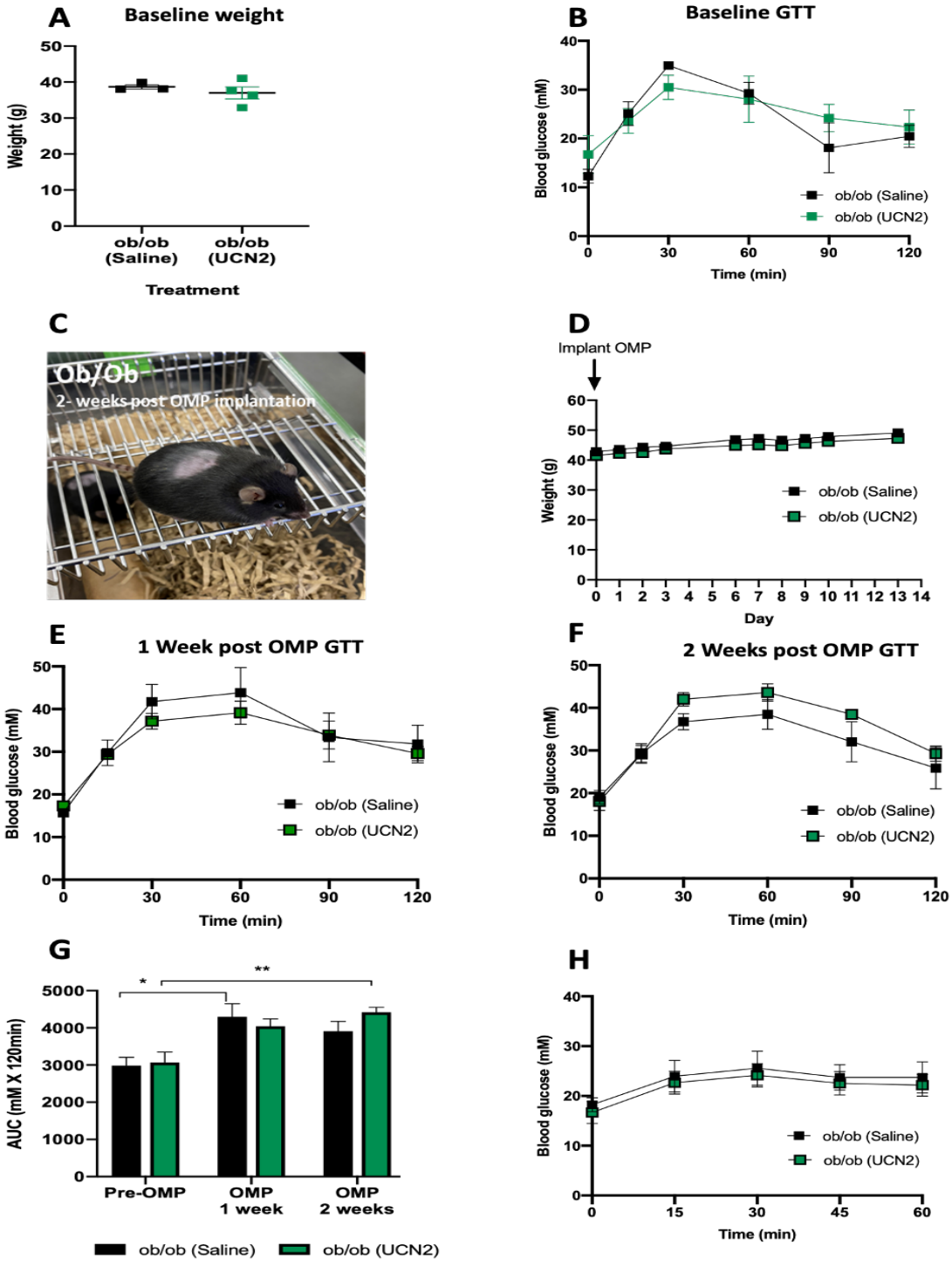

Figure 5-8 Chronic effects of UCN2 on glucose homeostasis in ob/ob mice. Dose 1: 5 pmol/h (Figure legend on following page)

Figure legend continued

Figure 5-8 Chronic effects of UCN2 on glucose homeostasis in ob/ob mice – Dose 1: 5 pmol/h. Male ob/ob mice were subcutaneously implanted with OMPs chronically infusing saline (black circles/bars) or UCN2 (green circles/bars). Baseline weight (A) and glucose tolerance (B) were recorded prior to OMP implantation. Image of mouse taken at the end of the study (C) and weight was monitored throughout the duration of the study (D). Fasted i.p. GTTs performed 1-week (E) and 2 -weeks (F) post peptide infusion. Representative AUC for baseline (pre-OMP), 1-week and 2 -week GTT compared in (G) and fasted i.p. ITT (performed on day 10 post infusion) shown in (H). Data are presented as mean ±SEM, n= 3-4 mice/group, *p< 0.05, **p<0.01 two-way RM-ANOVA/ two-way ANOVA followed by Sidak's / Tukey's multiple comparisons test.

Dose 2: 25 pmol/h

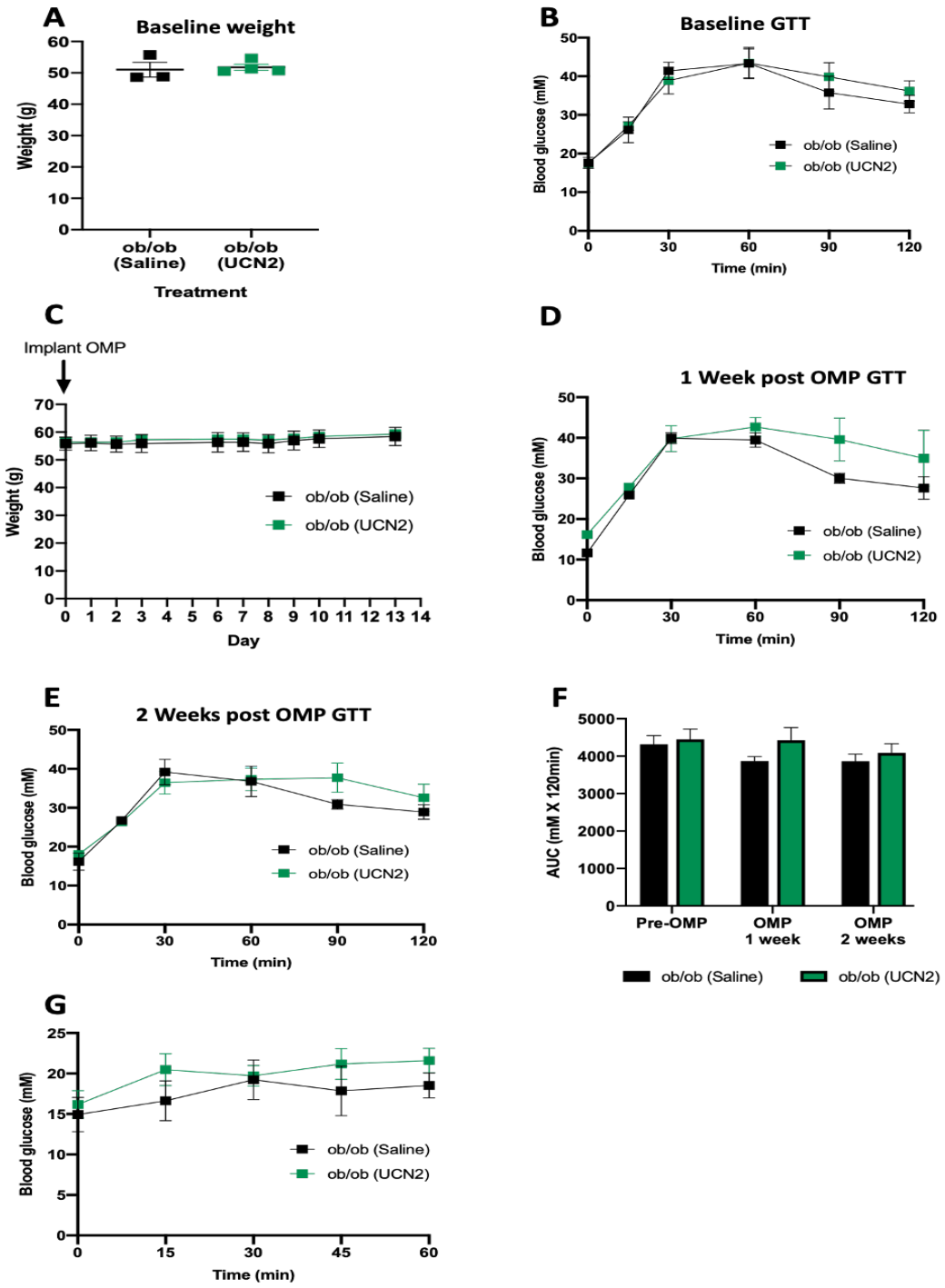

Figure 5-9 Chronic effects of UCN2 on glucose homeostasis in ob/ob mice. Dose 2: 25 pmol/h

(Figure legend on following page)

Figure legend continued

Figure 5-9 Chronic effects of UCN2 on glucose homeostasis in ob/ob mice – Dose 2: 25 pmol/h. Male ob/ob mice were subcutaneously implanted with OMPs chronically infusing saline (black circles/bars) or UCN2 (green circles/bars). Baseline weight (A) and glucose tolerance (B) were recorded prior to OMP implantation. Weight was monitored throughout the duration of the study (C). Fasted i.p. GTTs performed 1-week (D) and 2-weeks (E) post peptide infusion. Representative AUC for baseline (pre-OMP), 1-week and 2 -week GTT compared in (F) and fasted i.p. ITT (performed on day 10 post infusion) shown in (G). Data are presented as mean ±SEM, n= 3-4 mice/group, *p< 0.05, **p<0.01 two-way RM-ANOVA/ two-way ANOVA followed by Sidak's / Tukey's multiple comparisons test.

5.4 Discussion

Pregnancy represents a unique and dynamic state where there are rapid and reversible increases in β-cell number and function (Baeyens et al., 2016). Pregnancy, in particular rodent pregnancy, also represents an accessible paradigm that allows researchers to study the complex placental signals communicating with maternal β-cells to influence these adaptations. Signals involved in the islet adaptation to pregnancy may be potentially exploited therapeutically to both increase β-cell mass and increase β-cell function in GDM and also in the wider context of diabetes in general (Drynda et al., 2018; Moyce and Dolinsky, 2018). However, an endogenous pregnancy signal having beneficial effects on islet function does not necessarily guarantee that this peptide will have similar effects outside of pregnancy when given exogenously. Our studies thus far have identified a novel role for endogenous UCN2/CRHR2 signalling in supporting the functional adaptive β-cell response to pregnancy. To determine whether similar effects could be observed outside of pregnancy the capacity of β-cells to respond to UCN2 signalling *in vivo* was studied in obese, insulin resistant ob/ob mice. The preliminary findings from this study suggest that, at least in the ob/ob mouse model of impaired glucose homeostasis, UCN2 is unable to improve glucose tolerance as it does in mouse pregnancy. In fact, acutely or chronically administered UCN2 had either deleterious or no effects on glucose homeostasis respectively.

A bolus injection of UCN2 caused a worsening of glucose tolerance in both lean C57BL/6 and obese ob/ob mice. The dosing of UCN2 for this experiment was based on previous studies by Chen et al (2006) investigating the physiological function of UCN2 on glucose utilization and insulin sensitivity, particularly in skeletal muscle. This study administered synthetic UCN2 peptide (0.1 mg/kg of body weight, equivalent to our dose) to wild type (WT) or mutant (UCN2-null) male mice 20 minutes prior to fasted GTT. Glucose tolerance was worsened in mutant mice administered UCN2 compared to saline administered counterparts. In fact, saline-treated UCN2-null mice displayed superior glucose tolerance compared to all treatment groups, including WT-littermates and UCN2-null mice both administered UCN2 (A. Chen et al., 2006). This data would appear to parallel our observations and are also consistent with the idea that this acute dose of UCN2 negatively influences glucose tolerance. However, the equivalent data between WT mice administered UCN2 peptide compared to saline controls were not documented, making comparisons to the results we observed consistently in various mouse strains challenging. It was unexpected to observe such a potent worsening of glucose tolerance in both healthy lean C57BL/6 and obese ob/ob mice suggesting a mechanism was activated in both strains irrespective of their glucose control.

The dosing of UCN2 administered acutely to mice was approximately one-thousand times the circulating concentration of endogenous UCN2 measured during mouse pregnancy (Chapter 4

Figure 4-2D). As such these observations are unlikely to reflect β-cell mediated effects during the physiological scenario in pregnancy but may instead be the result of a pharmacological dose that would not be present endogenously. One potential explanation for the increased blood glucose in response to this supraphysiological dose of UCN2 is that it may have activated the sympathetic nervous system and/or the adrenal gland directly to stimulate catecholamine (i.e. adrenaline and noradrenaline) release. Both adrenaline and noradrenaline can exert complex direct and indirect actions on glucose metabolism with the primary objective of promoting an increase in blood glucose levels. The catecholamine response is mediated by plasma membrane GPCRs (adrenergic receptors) divided into α- and β- adrenoreceptors. The hyperglycaemic effect of both hormones results from a combination of directly stimulating glycogenolysis and gluconeogenesis via activation of β_2-adrenergic receptors in the liver and indirectly through enhancing glucagon secretion from pancreatic α-cells via α_2-adrenergic receptors (Clutter et al., 1988). Catecholamines further increase hyperglycaemia through the inhibition of insulin-mediated glucose uptake in insulin-sensitive tissues and through the inhibition of insulin secretion by pancreatic β-cells (Webber and MacDonald, 1993). Collectively, these responses would be consistent with the acute rise in blood glucose levels that were observed following acute UCN2 administration.

Although variable data exists concerning UCN2/CRHR2 -mediated sympathoadrenal activation and subsequent catecholamine release, *in vitro* data suggests that UCN2 may directly regulate catecholamine release from the adrenal gland. Cultured PC12 cells (a rat pheochromocytoma adrenal cell line) express type 2 CRHRs and when treated with UCN2 (0.1-10 nM for 2 h), significant stimulation of noradrenaline into the cell culture media was observed (Nemoto et al., 2005). Contrary to these observations, Dermitzaki et al, have reported that acute exposure of dispersed primary rat and human chromaffin cells (representing cells of the adrenal medulla) to UCN2 (at concentrations ranging between 0.1 nM – 0.1 μM) for 30 minutes, suppressed catecholamine secretion. However, the authors also demonstrated that longer exposure of rat chromaffin cells to CRHR2 agonists (48 h) did stimulate release of adrenaline and noradrenaline, suggesting a time-dependent fluctuation in catecholamine production (Dermitzaki et al., 2007).

Studies examining the effects of UCN2 on plasma catecholamine levels *in vivo* have demonstrated that in conscious sheep, bolus administration of UCN2 (25 and 100 μg) induced a significant rise, albeit short-lived, in plasma noradrenaline and a similar pattern was observed with plasma levels of adrenaline (Charles et al., 2010). Similar studies in sheep (bolus injection of 10, 50 and 100 μg UCN2) have however, previously shown no effects of UCN2 administration on plasma catecholamine levels (Rademaker Miriam T. et al., 2005) whereas intravenous infusion of UCN2 (100 μg) in humans has been shown to induce significant increases in plasma noradrenaline levels

(Davis et al., 2007). Despite these discrepancies, there is compelling evidence to suggest that UCN2 could regulate catecholamine release. If this hypothesis is correct it may also explain why ob/ob mice treated with UCN2, displayed a significantly decreased plasma insulin response to glucose challenge, as this is consistent with β-cells from ob/ob mice being more sensitive than lean mouse β-cells to the inhibitory effects of noradrenaline on glucose stimulated insulin release (Lindström, 2010; Tassava et al., 1992). Ideally plasma catecholamine levels following UCN2 administration to lean and ob/ob mice would have been measured in the present study as an index of global sympathetic nervous system activity, but unfortunately this was not possible due to laboratory closure. These additional experiments may be able to provide more clarity on these results.

It should also be noted that UCN2 is hypothesised to activate CRHR1 at higher doses, with a reported EC_{50} greater than 100 nM (Fekete and Zorrilla, 2007), in line with our pharmacological acute dosage. This may provide an alternative explanation for our results if they are not due to direct catecholamine action. Previous studies have shown that although UCN2 has no significant binding affinity to human CRHR1, cAMP stimulation studies revealed a weak agonist potency (> 1 μM) for UCN2 at recombinant CRHR1 (Hauger et al., 2003a). Given the role of CRHR1 activation on the corticotroph cells of the anterior pituitary on the HPA axis and subsequent cortisol release, it is possible that activation of pituitary CRHR1 could also contribute to the effects we observed on glucose tolerance.

Given that acute administration of pharmacological UCN2 failed to replicate the previously observed beneficial effects of UCN2 on islet function, subsequent experiments focused on harnessing the insulinotropic potential of UCN2 by attempting to mimic its chronically elevated physiological levels in the pregnant state. Thus, C57BL/6 and ob/ob mice were chronically infused with UCN2 peptide at two separate doses. These doses were calculated to generate steady state levels in the mouse within the range of the endogenous levels measured in mouse pregnancy and much lower than the pharmacological dose given acutely. Contrary to the adverse effects on glucose tolerance observed with acute UCN2 injection, chronic infusion of UCN2, at either dose had minimal effects on overall glucose homeostasis in either lean or obese animals. These data combined with the lack of effect seen with total CRHR blockade outside of pregnancy (Chapter 4) strongly suggests that something specific to the pregnancy environment enables UCN2 to facilitate the pancreatic islet adaptations only during gestation.

It is unsurprising that no significant improvements in glucose tolerance were observed in lean healthy C57BL/6 mice, given these mice already have healthy glycaemic control. Interestingly, chronic low-dose UCN2 treatment did appear to transiently impair insulin sensitivity, though this effect was only significant 15 minutes post-insulin administration. The effect was reversed at the

slightly higher dose, where insulin sensitivity appeared to then be improved. Given the mild and inconsistent nature of this result between doses, it is difficult to be certain that this is a genuine result as opposed to an experimental artifact. However, one of the primary mechanisms UCN2 is reported to influence glucose tolerance is via modulating skeletal muscle insulin sensitivity, though there are inconsistencies in the literature. Chen et al found that UCN2 null mutant mice display increased glucose uptake in skeletal muscle, implying that endogenous UCN2 inhibits insulin signalling in skeletal muscle (A. Chen et al., 2006). More recently others have claimed that UCN2 enhances glucose disposal and acts as an insulin sensitizer in skeletal muscle (Borg et al., 2019; Gao et al., 2019, 2016). These *in vivo* studies utilising adeno-associated virus UCN2 gene transfer systems, or treatment with modified UCN2 peptide in animal models of diabetes, demonstrated increased glucose infusion rates throughout hyperinsulineamic-euglycaemic clamp studies in addition to increased GLUT-4 translocation in skeletal muscle cells *in vitro*, supporting the theory of enhanced insulin signalling/sensitivity with UCN2 treatment (Borg et al., 2019; Gao et al., 2019, 2016). Our insulin tolerance tests in lean mice chronically administered UCN2 do not conclusively support either hypothesis, with the low-dose results supporting an inhibitory effect of UCN2 and the higher dose suggesting a sensitising effect, if anything. However, neither of the transient changes in insulin tolerance were reflective of any alterations to overall glucose tolerance and would require further investigation given the limited number of biological replicates studied at either dose.

Similarly, in our ob/ob mouse studies, no consistent metabolic phenotype was evident following chronic UCN2 infusion suggesting that the beneficial effects of UCN2 on islet function are specific to pregnancy and are not operational in other models of insulin resistance. The first cohort of ob/ob mice studied using the lower dose of UCN2, did display a significant worsening of glucose tolerance at 2 -weeks post peptide infusion, as quantified by AUC in comparison to pre-OMP (baseline). However, a similar phenomenon was also exhibited by control mice who also displayed worse glucose tolerance in the 1- week (post OMP implant) GTT than they did in the pre-surgery baseline GTT. This most probably reflects the progressive insulin resistance which is recognised to develop with the age of the mice and increasing obesity (Tomita, 2016) as opposed to any direct effects of the peptide itself on glucose tolerance. Interestingly, in the second cohort, with a slightly higher dose of UCN2, no observable phenotypes in glucose or insulin tolerance were detected, although those mice treated with UCN2 typically had higher glucose levels throughout the study. The second cohort of mice were slightly older than the first cohort so this could explain why the same pattern of impairment to glucose tolerance was not as striking to that of the first cohort. Although unlikely, it cannot be ruled out that the two different doses of UCN2 may have different/opposing effects on glucose tolerance. As such, it would be important to confirm this in repeated studies using age-matched mice for both doses of UCN2.

The lack of any observable effect of chronic UCN2 on glucose tolerance in ob/ob mice is disappointing and suggests a pregnancy-specific effect. However, the effects of UCN2 that we saw in pregnancy were relatively mild and it is also possible that other factors within the obese ob/ob environment may have masked any beneficial effects on the islets. The obesogenic environment is commonly associated with increased adiposity and consequently alterations in hormone secretion from adipose tissue (Ouchi et al., 2011). Recently, differential dysregulation of UCN3 levels have been reported in obesity and T2DM. In a cohort of human subjects who were overweight, either with or without T2DM, UCN3 mRNA expression and protein in subcutaneous adipose tissue (SAT) increased with body weight. Conversely, UCN3 plasma levels decreased in overweight subjects (Kavalakatt et al., 2019). Although visceral fat biopsies may have been more clinically relevant in the context of obesity, the study does highlight the emerging role of UCNs in energy homeostasis, particularly within key metabolic tissues. It is therefore possible that dysregulation of these systems may also be altered in metabolic syndromes such as obesity. Whether or not a similarly altered profile of UCN2 is present in tissues/plasma in obesity is yet to be characterised. It is possible that an already altered profile of endogenous UCN2 exists in the ob/ob mice, but this was not determined in our studies. If endogenous levels of UCN2 are elevated in ob/ob mice, this may have influenced both our acute and chronic studies.

As mentioned in the introduction, although ob/ob β-cells are secreting insulin at a high capacity, they are still able to respond to most stimulators and inhibitors of insulin release and are often more sensitive to neural regulation of insulin secretion (Tassava et al., 1992). Therapeutic strategies to enhance insulin secretion have thus been examined and shown efficacy in the ob/ob mouse as a model of obesity, insulin resistance and T2DM (Skow et al., 2016). The potentiating effect of UCN2 on insulin release has been shown *in vitro* (Chapter 3) and is consistent with the impairment to glucose tolerance upon selective CRHR2 blockade during pregnancy (Chapter 4). It was therefore expected that the UCN2-mediated potentiation of insulin release could improve glycaemic control in ob/ob mice. Despite other modified insulin secretagogues exhibiting enhanced anti-hyperglycaemic activity and insulin-releasing action *in vivo* in ob/ob mice (O'Harte et al., 2000), we were unable to detect any positive effects of UCN2 on glycaemic control. Given the hyperinsulinemia displayed by these mice at baseline, it is possible that even if UCN2 did cause modest improvements to β-cell insulin secretion in response to glucose, it would be masked by the large amount of insulin already being secreted in response to the marked peripheral insulin resistance. Whereas other incretin-based therapies have proved promising in these models, these hormones most likely have pleiotropic effects not just limited to enhancing insulin secretion which in combination are able to have the net effect of improving the dysglycaemia in ob/ob mice. Perhaps a different approach, such as infusing the CRHR2 selective antagonist into ob/ob mice to see whether this worsens glucose tolerance, or even a

different mouse model, possibly using a milder metabolic phenotype induced by high-fat diet (HFD), may have enabled us to circumvent the limitations discussed above.

Attempting to model or exploit mechanisms in obesity or pregnancy poses many challenges, especially as they both represent complex states. It is unlikely that one strategy or even a single hormone will provide the therapeutic efficacy to rectify the defects associated with the dysglycaemia in obesity or impaired β-cell compensatory mechanisms which may be present in gestational diabetes. Nevertheless, signalling molecules identified to have influential roles in critical pathways can be extremely valuable as candidates for clinical translation. Even if these signals are not suitable for therapeutic administration, they may potentially serve as predictive markers to indicate those at risk to pathological processes. In the case of pregnancy, measuring these markers may identify those more susceptible to glucose intolerance or GDM. The next chapter will thus focus on the translational relevance of CRH peptides, particularly UCN2, in human gestation investigating correlations between CRH peptide levels during pregnancy and degrees of glucose intolerance/ GDM.

Chapter 6

Chapter 6 Correlations between circulating CRH/ UCNs and glucose tolerance in pregnant women

Chapter snapshot

Chronic administration of UCN2 to obese ob/ob mice did not improve glucose tolerance by mimicking the positive effects of placental UCN2 during pregnancy. This suggests that the effect of UCN2 on islet function may be a unique mechanism to pregnancy.

Although UCN2/CRHR2 signalling appears to be the probable mechanism to facilitate enhanced glucose-induced insulin secretion during mouse pregnancy, whether this is true for human pregnancy is yet to be determined.

Therefore, the aim of this chapter was to focus on the translational relevance of CRH peptides, particularly UCN2, in human gestation by investigating correlations between CRH/UCN peptide levels and glucose tolerance in pregnant women.

6.1 Introduction

Animal models (particularly rodent models) have been instrumental in furthering our understanding of the physiology of pregnancy, especially the characterisation of β-cell adaptations in response to the increased maternal insulin demand and the signals involved in regulating them. However, as Baeyens and colleagues (2016) highlight in their recent review, caution should be taken when trying to extrapolate the results of studies performed in rodent models to humans (Baeyens et al., 2016). Many aspects of pregnancy physiology differ between rodents and humans, most notably the gestation length and typical number of foetuses. Differences are also displayed in placental protein expression and secretion profiles along with the regulatory mechanisms of parturition (Andersen et al., 2018; Goyvaerts et al., 2016). As discussed in the general introduction (Chapter 1) the CRH system has long been recognised to have a differential role in placental mechanisms controlling pregnancy and labour, particularly between anthropoid primates and rodents (Bernal, 2001; Power and Schulkin, 2006; Robinson et

al., 1989). At least two patterns of placental CRH expression over gestation have been reported among anthropoid species (Power et al., 2010; Power and Schulkin, 2006). In humans, circulating CRH is undetectable throughout the first trimester but increases exponentially from the second trimester to peak at term (Campbell et al., 1987; McLean et al., 1995; Sasaki et al., 1988, 1987). Following parturition and delivery of the placenta, circulating CRH rapidly returns to undetectable levels. It is now known that the placenta is the major source of the circulating CRH, reflecting a more than 20-fold increase in CRH mRNA which parallels with the rise in placental CRH peptide content and secretion profile (Frim et al., 1988; Karteris et al., 2001; Power et al., 2006). This pattern of CRH secretion and the ability of CRH to stimulate myometrial contractility, in part via promoting a cascade of inflammation in the uterus, is consistent with CRH acting as a "placental clock", controlling the onset of labour (Grino et al., 1987b; McLean et al., 1995; Rosen et al., 2015; Saijonmaa et al., 1988). As such, in humans, abnormally high placental expression of CRH is associated with preterm labour (McLean et al., 1995; Wadhwa et al., 2004; Warren et al., 1992). Thus, placental CRH appears to be a distinct adaptive function/parturition mechanism to humans. The lack of detectable placental/maternal circulating CRH in rodents has therefore suggested that the CRH system does not play an important role in modulating labour in these species (Power and Schulkin, 2006; Robinson et al., 1989). Correspondingly, pregnancies from CRH-null mutant mice demonstrate normal spontaneous labour and deliver at the same time as wild-type controls suggesting that CRH is not essential for the normal timing of parturition in mice (Bernal, 2001; Muglia et al., 1995). In fact, progesterone withdrawal triggered by an increase in prostaglandin signalling is considered to be the principal mechanism for the onset of normal murine parturition (Ratajczak and Muglia, 2008). The functional significance of the difference of placental CRH between species is not yet understood but it is possible that the capacity of the primate placenta to synthesise CRH is much higher than that of the murine placenta (Bernal, 2001; Perkins and Linton, 1995). Nevertheless, there are still similarities between rodent and human pregnancy physiology including the fundamental responses of pancreatic islets to the pregnant environment (i.e. enhanced insulin secretion and β-cell mass expansion). However, the complete characterisation of these responses in both rodents and humans remains incomplete.

Studying gestational-induced islet adaptations in humans is challenging due to the difficulty in obtaining pregnant pancreatic autopsy specimens and the inability and ethical restrictions of studying islet anatomy *in vivo.* Despite this, two autopsy series of pregnant human pancreatic tissue have been analysed, revealing some similarities but also potentially key differences in the mechanisms responsible for β-cell adaptations between rodents and humans (Butler et al., 2010; Genevay et al., 2010; Van Assche et al., 1978). It is well known that rodents undergo a rapid and substantial expansion of β-cell mass in addition to significant increases in insulin output during pregnancy (Baeyens et al., 2016). Human β-cells also demonstrate considerable functional improvement but it has been suggested that the increase in β-cell mass is less extensive than that

in rodents: 3-5-fold in rodents versus 1.4-2.4-fold in humans (Butler et al., 2010; Genevay et al., 2010). The major mechanisms facilitating β-cell mass expansion in rodents are hypertrophy and hyperplasia (increase in β-cell size and β-cell replication respectively) (Rieck and Kaestner, 2010). Although the earlier report by Van Assche proposed the marked enlargement of islets in pregnant women was mainly due to hyperplasia of the β-cells (Van Assche et al., 1978), the latest report by Butler et al (2010) suggests that the increase in the β-cell mass may be attributable to alternative mechanisms. Despite a moderate increase in human β-cell fractional area, no evidence of β-cell proliferation or increases in β-cell size were detected between age-matched pregnant and non-pregnant women (Butler et al., 2010). Instead it was observed that in pregnant human specimens there was an increase in scattered β-cells and duct cells positive for insulin, suggestive of β-cell neogenesis as opposed to the β-cell hyperplasia and hypertrophy characterised in rodent pregnancy (Butler et al., 2010).

Given that there may be dissimilarities in the cellular mechanisms responsible for β-cell mass expansion between humans and rodents, it is equally possible that the signals regulating the islet adaptations between the species may also differ. For example, lactogenic hormones are suggested to have a less pronounced contribution to human β-cell adaptations than that demonstrated in rodent pregnancy. Although placental lactogen (PL), prolactin (PRL) and β-cell prolactin receptor (PRLR) signalling have been extensively studied in rodents and identified as key hormones inducing islet adaptations in rodent pregnancy, conflicting data exist regarding the effects of lactogen treatment on human islets. Studies conducted by Brelje et al (1993) demonstrated that adult human islets cultured with human placental lactogen (hPL) or human prolactin (hPRL) for 8 days increased glucose-stimulated insulin secretion (GSIS). Evidence of increased proliferation as measured by BrdU incorporation was also observed in human islets cultured with lactogenic hormones (Brelje et al., 1993). However, later studies using dispersed human islets have failed to detect increases in β-cell proliferation when treated with human prolactin (Chen et al., 2015). One plausible explanation for these discrepant findings between the effects of lactogens on rodent islets compared to human islets may be attributable to the gene expression differences between mouse and human β-cell PRLRs. In comparison to human β-cells, the PRLR is abundantly expressed in mouse β-cells (Baeyens et al., 2016; Benner et al., 2014; Xin et al., 2016). This may indicate a lesser role for lactogenic signalling in human islet adaptations to pregnancy. However, it is most likely that lactogenic hormones do play some role in human β-cell compensation though the mechanistic pathways may not necessarily be the same as those in rodents.

The differences between rodent and human pregnancies/β-cell biology therefore need to be considered when investigating whether signals/mechanisms established in rodents apply to human pregnancy. Thus, whilst our current data is consistent with a role for endogenous UCN2/CRHR2

signalling in supporting β-cell adaptations during mouse pregnancy, it is important to confirm whether our observations from our mouse studies translate to humans. One approach to do this is to investigate whether circulating levels of the CRH peptides correlate with glucose intolerance or GDM in pregnant women. This chapter addresses the hypothesis that circulating levels of CRH peptides will correlate to glucose homeostasis in human pregnancy with women with GDM displaying lower circulating levels of all or some of CRH peptides.

6.2 Materials and Methods

6.2.1 Study approval

The research in humans was conducted in accordance with the Declaration of Helsinki (2013) and was approved by the London-Westminster Research Ethics Committee (13/LO/0539). Written informed consent was obtained from all participants.

6.2.2 Collection, storage and processing of human samples

Blood samples were collected by a clinical research nurse at King's College Hospital from pregnant women undergoing a routine fasted 2-hour 75 g oral glucose tolerance test (OGTT) between 26- and 34- weeks' gestation (described in section 2.8). Venous blood samples were collected into tubes containing Fluoride EDTA for plasma or SST collection tubes for serum samples (BD Vacutainer Blood Collection Tubes). Samples were taken at baseline as well as at 10, 60 and 120 minutes for the measurement of plasma glucose and serum insulin. Additional blood samples were taken prior to glucose consumption, collected into BD Vacutainer Blood Collection Tubes containing EDTA and Trasylol for the measurement of circulating CRH/UCNs and other hormones. All samples were processed accordingly to obtain plasma (centrifuged at 855 *g*, 10 mins, 4ºC) or serum (allowed to stand for a minimum of 20 minutes at room temperature before centrifugation at 855 *g*, 10 mins, 4ºC). Plasma glucose was measured immediately using a YSI 23000 Stat Analyser (YSI Life Sciences) or samples were stored at -80ºC until assay. Serum insulin was measured using a commercially available ELISA kit (10-1113-01, Mercodia, Sweden) according to the manufacturer's instructions. Participants were diagnosed with GDM according to the International Association of Diabetes and Pregnancy Study Groups (IADPSG) criteria (Consensus Panel, 2010). The IADPSG is an umbrella organization formed in 1998 to encourage and facilitate the collaboration of international research and enhance education in the field of diabetes in pregnancy. Based on associations between degrees of maternal glucose intolerance and risks of adverse perinatal outcomes reported in the Hyperglycaemia and Adverse Pregnancy Outcome (HAPO) study (HAPO Study Cooperative Research Group et al., 2008), the IADPSG consensus panel devised GDM diagnostic threshold recommendations as follows: fasting plasma glucose ≥ 5.1 mmol/l, 1-hour plasma glucose ≥10.0 mmol/l or 2-hour plasma glucose ≥8.5 mmol/l (Consensus Panel, 2010). One or more of these values from a 75 g OGTT must be met for diagnosis of GDM.

6.2.3 Homeostasis Model Assessment (HOMA) indices

Physiological techniques employed in clinical studies for the measurement of insulin sensitivity and β-cell function *in vivo* include: hyperinsulineamic-euglycaemic clamps, glucose tolerance tests and mathematical modelling derived from computer based solutions based on the kinetics of

insulin and glucose (Pacini and Mari, 2003; Song et al., 2007). Clamp techniques require expertise, are time and money consuming and therefore their use is limited in medium -large scale studies. However, mathematical modelling of the glucose-insulin homeostatic system offers a simpler, inexpensive and robust clinical tool for the estimation/assessment of insulin resistance and pancreatic β-cell function. Homeostasis Model Assessment (HOMA) was first developed in 1985 by Matthews et al (Matthews et al., 1985) and is a mathematical model based on fasting plasma glucose and insulin concentrations, providing reliable surrogate markers of an individual's degree of insulin resistance (HOMAR-IR) and level of pancreatic β-cell function (HOMA-%β) (Matthews et al., 1985) (see Figure 6-1 for simplified equations of both indices). Generally, the higher the HOMA-IR readout (normal IR is defined as 1), the more insulin resistant an individual is whereas for HOMA-%β, a lower readout (normal β-cell function defined as 100%) indicates greater β-cell dysfunction (Al-Mahmood et al., 2006; Imamura et al., 2013). It is recommended that both indices should be reviewed collectively and not in isolation to avoid making inappropriate conclusions about β-cell function not in the context of an individual's insulin sensitivity (Wallace et al., 2004). Since the initial development of HOMA (1985), the model has been updated and recalibrated (HOMA2) to provide a more accurate index of insulin resistance and β-cell function (i.e. HOMA2-IR and HOMA2-%β respectively) (Basukala et al., 2018; Levy et al., 1998). The HOMA2 calculator (released in 2004) is available to researchers and provides quick and easy access to HOMA2 calculations. HOMA2-IR and HOMA2-%β were calculated for the cohort of pregnant women included in this chapter using the HOMA2 calculator (htt://www.dtu.ox.ac.uk/homacalculator).

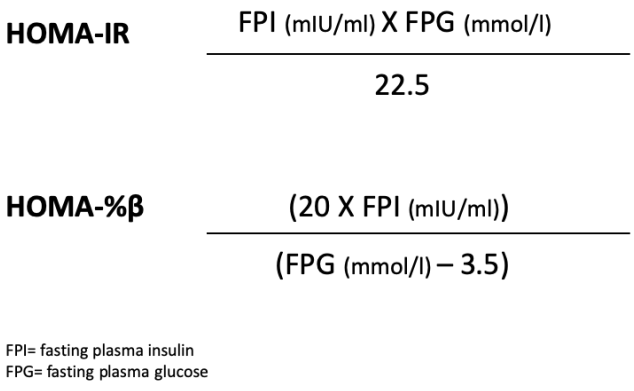

Figure 6-1 Approximating formula for calculating HOMA-IR and HOMA-%β based on the original computational method.

6.2.4 Preparation and assay of human samples for CRH peptides

Basal plasma samples collected during the OGTT that had been stored at -80ºC were subsequently assayed for CRH, UCN1, UCN2 and UCN3 using commercially available ELISA kits as detailed in Table 6-1 below. A total of 63 samples were blindly assayed in duplicate according to the manufacturer's instructions and absorbances measured at 450 nm using a PHERAstar FS microplate reader (BMG Labtech, UK).

Peptide	Supplier	Product No.
CRH	Cloud-Clone Corp, USA	CEA835Hu
UCN1	Cloud-Clone Corp, USA	SEA231Hu
UCN2	Cloud-Clone Corp, USA	SEC585Hu
UCN3	Cloud-Clone Corp, USA	CED140Hu

Table 6-1 Supplier details for CRH and Urocortin ELISA kits.

6.3 Results

6.3.1 Circulating CRH/UCN levels in pregnant women with and without GDM

GDM was diagnosed in participants who had undergone a routine 2 h 75 g OGTT during pregnancy (according to IADPSG criteria described in the materials and methods above). Of the total 63 samples assayed, 23 women had GDM and 40 did not. There were no differences in participant characteristics (including age, gestation and ethnicity) between healthy women and women with GDM however, those with GDM had a significantly higher BMI compared to healthy controls (Healthy vs GDM, 29.36±1.09 vs 33.30±1.33 kg/m^2 $p<0.05$) (see supplementary Table 6-5 for breakdown of patient characteristics). No significant differences in pregnant circulating levels of UCN2, UCN3 and CRH were measured between healthy women and women with GDM (as shown in (Figure 6-2) (UCN2: Healthy vs GDM, 14.71±1.05 vs 15.71±1.52 pg/ml, p=0.595; UCN3: 62.57±7.99 vs 81.24±14.15 pg/ml, p=0.258; CRH: 39.43±1.72 vs 40.50± 3.73 pg/ml, p=0.798). Of the peptides measured, UCN3 displayed the highest plasma levels in pregnant women. Unfortunately, plasma levels of UCN1 were below the lower detectable limits of the ELISA for all samples. As such no UCN1 results are included in this chapter but the plasma levels of UCN1 being undetectable in pregnant women suggests that this hormone does not play an important role in regulating glucose homeostasis during pregnancy.

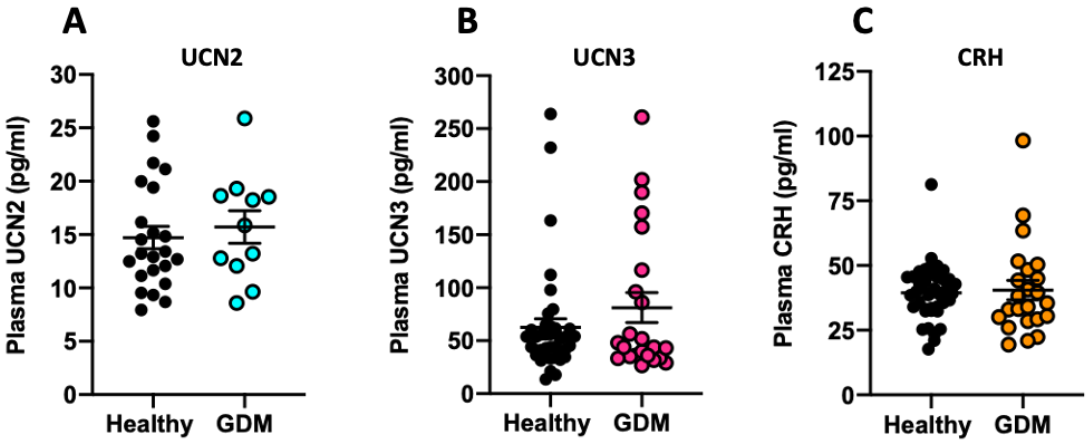

Figure 6-2 Circulating plasma levels of CRH/UCNs in pregnant women. Plasma UCN2 (A), UCN3 (B) and CRH (C) were measured in pregnant women (between 26-34 weeks' gestation) without GDM (healthy-black circles) and with GDM (cyan, pink and orange circles, respectively). Data are presented as individual values per participant with mean ±SEM indicated by intersecting lines, n= 11- 40 per group. Data were statistically analysed using two-tailed t-test, p >0.05 for all peptides.

6.3.2 Relationship between UCN2 and glucose tolerance in pregnant women with and without GDM

In the cohort of pregnant women sampled, Pearson's correlation coefficients were examined between plasma UCN2 and; 1) serum insulin levels throughout the OGTT (Figure 6-3), 2) plasma glucose during OGTT (Figure 6-4) and 3) calculated surrogate indices for insulin resistance (HOMA2-IR) and β-cell function (HOMA2-%β) (Figure 6-5). For each peptide correlation coefficients were calculated for the entire cohort (i.e. all pregnant women both with and without GDM) as well as for women with and without GDM separately for all parameters and time points throughout the OGTT (Table 6-2). Correlation coefficient lines were only displayed on graphs where a trend (defined as $p < 0.2$) was observed in either control women or those diagnosed with GDM.

No significant correlations were observed between UCN2 and either basal fasting insulin or insulin post-glucose when looking at the full cohort of women (Figure 6-3A-E & Table 6-2). However, when looking specifically in women diagnosed with GDM a moderate-strength positive correlation between UCN2 and serum insulin was observed at 60 minutes post-glucose load with a trend towards significance ($r = 0.566$; $p = 0.07$) (Figure 6-3C). There was a similar moderate-strength positive correlation between UCN2 and overall glucose-stimulated insulin levels, as assessed by insulin AUC, for women with GDM only, although again this did not reach statistical significance ($r = 0.461$; $p = 0.154$) (Figure 6-3E).

Although no correlations were observed between UCN2 and plasma glucose at baseline and for the first hour of the OGTT (Figure 6-4A-C), a weak positive correlation was observed in the entire cohort and healthy women only by 2-hours (Figure 6-4D). Correlation analysis between UCN2 and HOMA2 indices revealed no significant correlations between UCN2 and HOMA2-IR (Figure 6-5A). In women with GDM a moderate-strength positive correlation between UCN2 and HOMA2-%β was displayed ($r =0.486$; $p = 0.130$) consistent with the correlation between UCN2 and glucose-induced insulin secretion in this group discussed above.

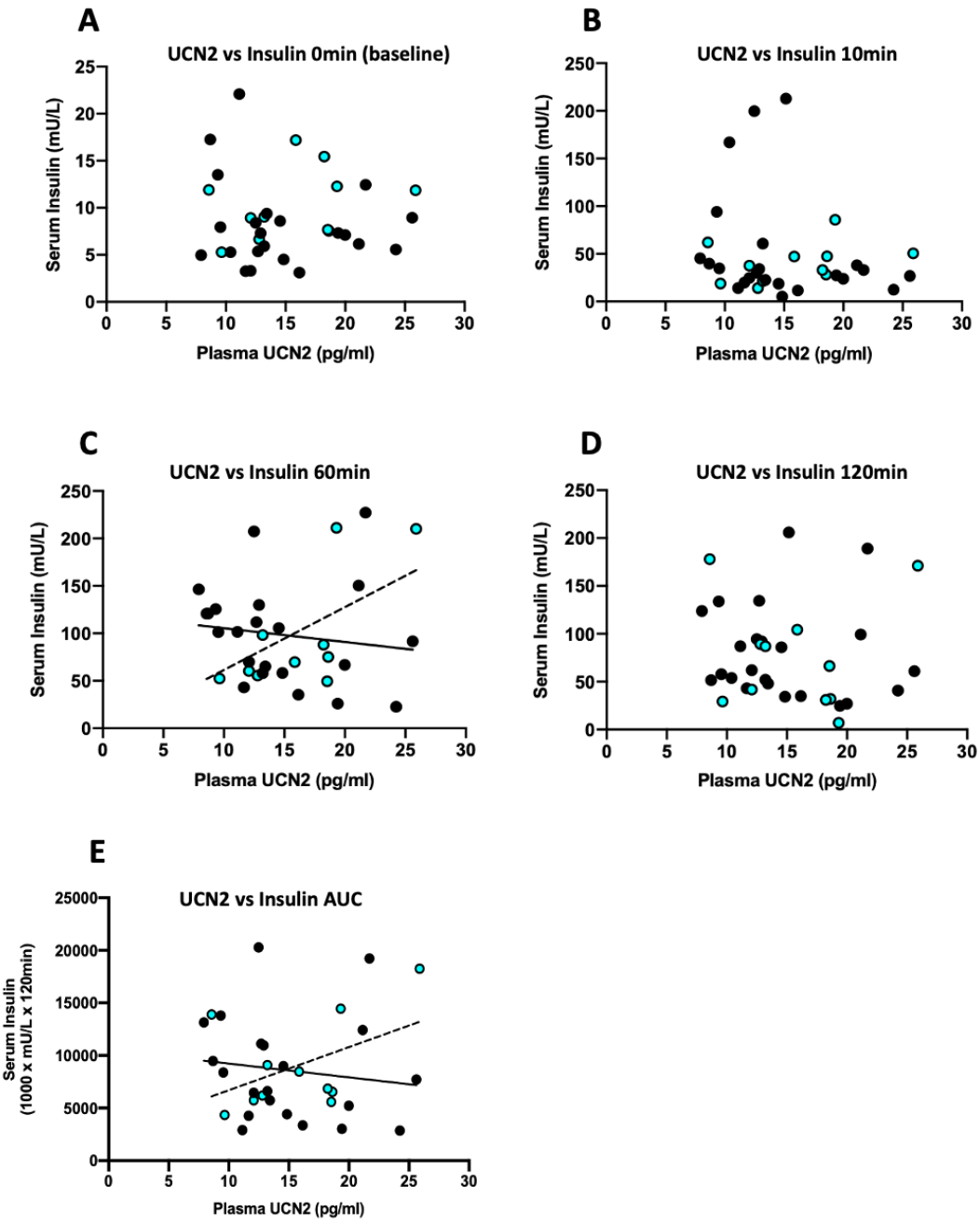

Figure 6-3 Relationship between UCN2 and serum insulin during an OGTT in pregnant women. Measurement of plasma UCN2 levels, fasted insulin (A) and insulin response to oral glucose in pregnant women during a routine 2-h 75 g OGTT between 26-34 weeks' gestation. Serum insulin measurements at 10 minutes (B), 60 minutes (C) and 120 minutes (D) following oral glucose with subsequent total insulin secreted (Area under the curve (AUC)) during the course of the OGTT (E) are shown. Women diagnosed with GDM are represented by cyan circles and women without GDM represented by black circles. Pearson's correlation coefficient was used for analysing correlation data. Dashed black line represents correlation coefficient line for women with GDM and solid black line for healthy women.

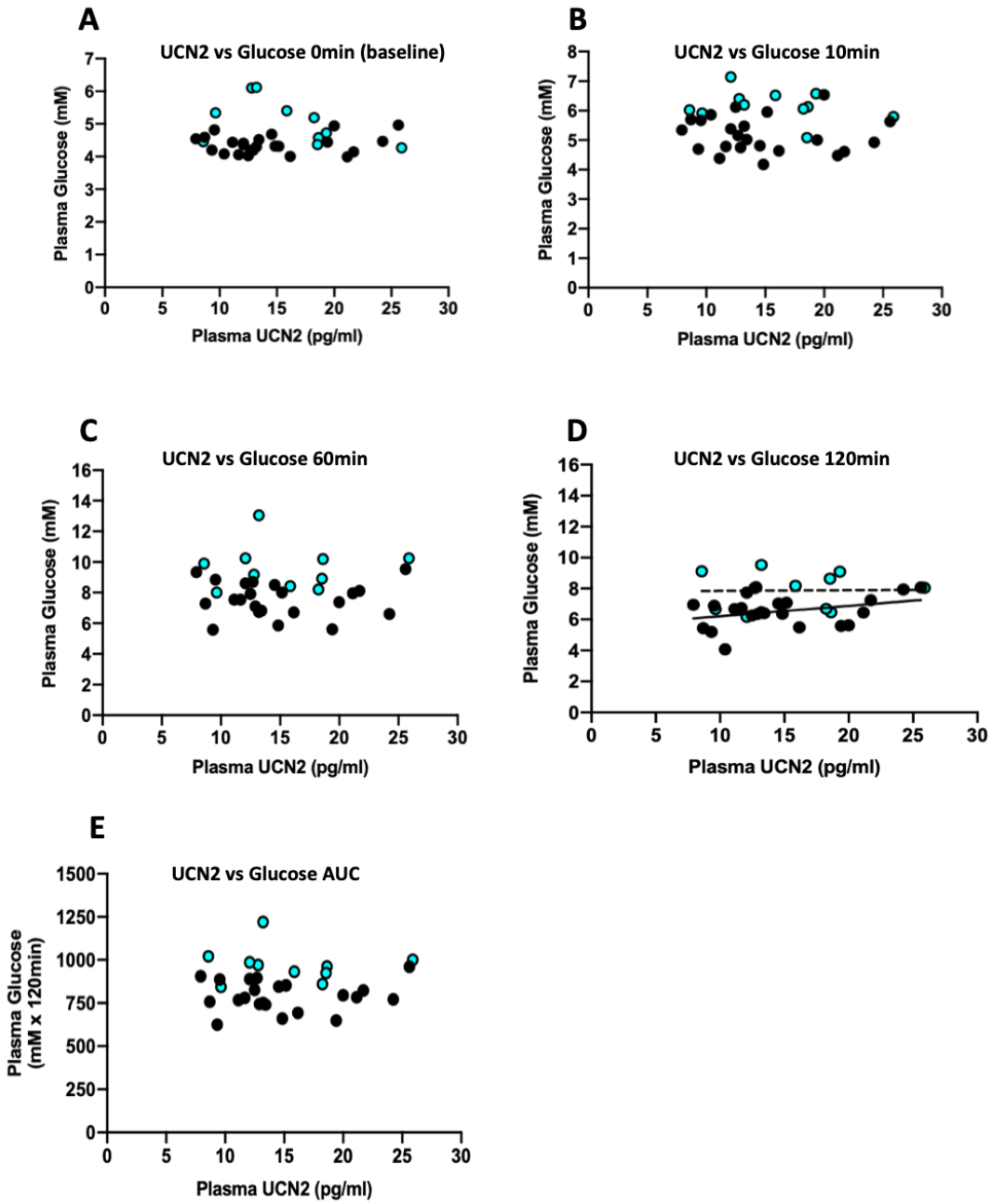

Figure 6-4 Relationship between UCN2 and plasma glucose during an OGTT in pregnant women. Measurement of plasma UCN2 levels, fasted plasma glucose (A) and glucose response during a routine 2-h 75 g OGTT in pregnant women between 26-34 weeks' gestation. Plasma glucose measurements at 10 minutes (B), 60 minutes (C) and 120 minutes (D) following oral glucose and glucose AUC (E) are shown. Women diagnosed with GDM are represented by cyan circles and women without GDM represented by black circles. Pearson's correlation coefficient was used for analysing correlation data. Dashed black line represents correlation coefficient line for women with GDM and solid black line for healthy women.

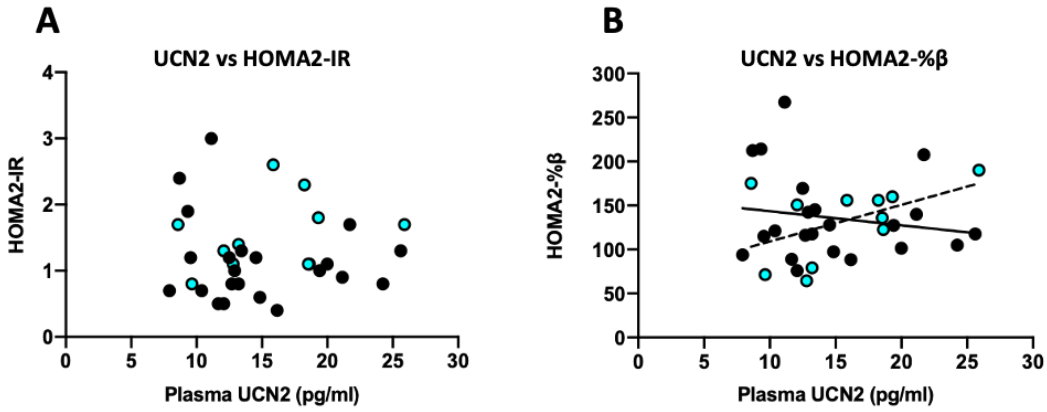

Figure 6-5 Relationship between UCN2 and HOMA2-IR and HOMA2-%β. HOMA2-IR (A) and HOMA2-%β (B) were calculated from fasted insulin and glucose measurements obtained from pregnant women undergoing a routine 2-h 75 g OGTT between 26- 34 weeks' gestation and compared to plasma UCN2 levels. Women diagnosed with GDM are represented by cyan circles and women without GDM represented by black circles. Pearson's correlation coefficient was used for analysing correlation data. Dashed black line represents correlation coefficient line for women with GDM and solid black line for healthy women.

UCN2	All pregnant women (n=34)		Healthy women (n=23)		Women with GDM (n=11)	
	Pearson's R correlation coefficient	p value	Pearson's R correlation coefficient	p value	Pearson's R correlation coefficient	p value
Insulin correlations						
UCN2 vs. Insulin 0 min	-0.003	0.988	-0.156	0.490	0.315	0.346
UCN2 vs. Insulin 10 min	-0.159	0.370	-0.239	0.272	0.323	0.332
UCN2 vs. Insulin 60 min	0.105	0.566	-0.137	0.554	0.566	0.070
UCN2 vs. Insulin 120 min	-0.041	0.816	-0.071	0.748	0.023	0.947
UCN2 vs. Insulin AUC	0.049	0.789	-0.134	0.564	0.461	0.154
Glucose correlations						
UCN2 vs. Glucose 0 min	-0.032	0.860	0.173	0.430	-0.370	0.262
UCN2 vs. Glucose 10 min	-0.044	0.806	-0.078	0.724	-0.283	0.399
UCN2 vs. Glucose 60 min	0.022	0.904	-0.013	0.954	0.009	0.981
UCN2 vs. Glucose 120 min	0.243	0.167	0.345	0.107	0.017	0.960
UCN2 vs. Glucose AUC	0.042	0.821	0.071	0.753	-0.084	0.817
Homeostatic model assessment (HOMA) correlations						
UCN2 vs. HOMA2-IR	0.008	0.966	-0.148	0.511	0.287	0.393
UCN2 vs. HOMA2-%β	0.020	0.912	-0.167	0.458	0.486	0.130

Table 6-2 Pearson's correlation coefficient data between UCN2 and insulin and glucose responses during OGTT in pregnant women. Correlations and statistical analysis for the entire cohort (All pregnant women) and women without (Healthy) and with GDM are shown.

6.3.3 Relationship between UCN3 and glucose tolerance in pregnant women with and without GDM

In the cohort of pregnant women sampled, Pearson's correlation coefficients were examined between plasma UCN3 and; 1) insulin response to oral glucose (Figure 6-6), 2) plasma glucose during OGTT (Figure 6-7) and 3) calculated surrogate indices for insulin resistance (HOMA2-IR) and β-cell function (HOMA2-%β) (Figure 6-8). Correlation coefficients were calculated for the entire cohort (i.e. all pregnant women both with and without GDM) as well as for women with and without GDM separately (Table 6-3).

Correlation coefficients for the entire cohort revealed no significant correlations between UCN3 and overall insulin responses throughout the OGTT (UCN3 vs Insulin AUC: $r = -0.022$; $p = 0.865$) (Figure 6-6E) though some modest correlations between UCN3 and plasma glucose were observed (Figure 6-7). When looking at the groups of pregnant women separately, a positive, albeit weak, correlation was observed between UCN3 and basal insulin ($r = 0.342$; $p = 0.111$) (Figure 6-6A). Associations were also displayed between UCN3 and plasma glucose which were dependent on whether the women were healthy or had GDM. For example, a weak negative correlation was observed between plasma UCN3 and basal glucose levels in healthy women ($r = -0.266$; $p=0.097$) (Figure 6-7A). However, at 10 minutes post oral glucose, a weak positive correlation was seen between UCN3 and plasma glucose in women with GDM which was absent in healthy women ($r = 0.292$; $p=0.177$) (Figure 6-7B). Additionally, there was also a weak positive correlation between UCN3 and HOMA2-IR, with a trend towards significance in women with GDM compared to healthy controls. ($r = 0.365$; $p = 0.087$) (Figure 6-8A).

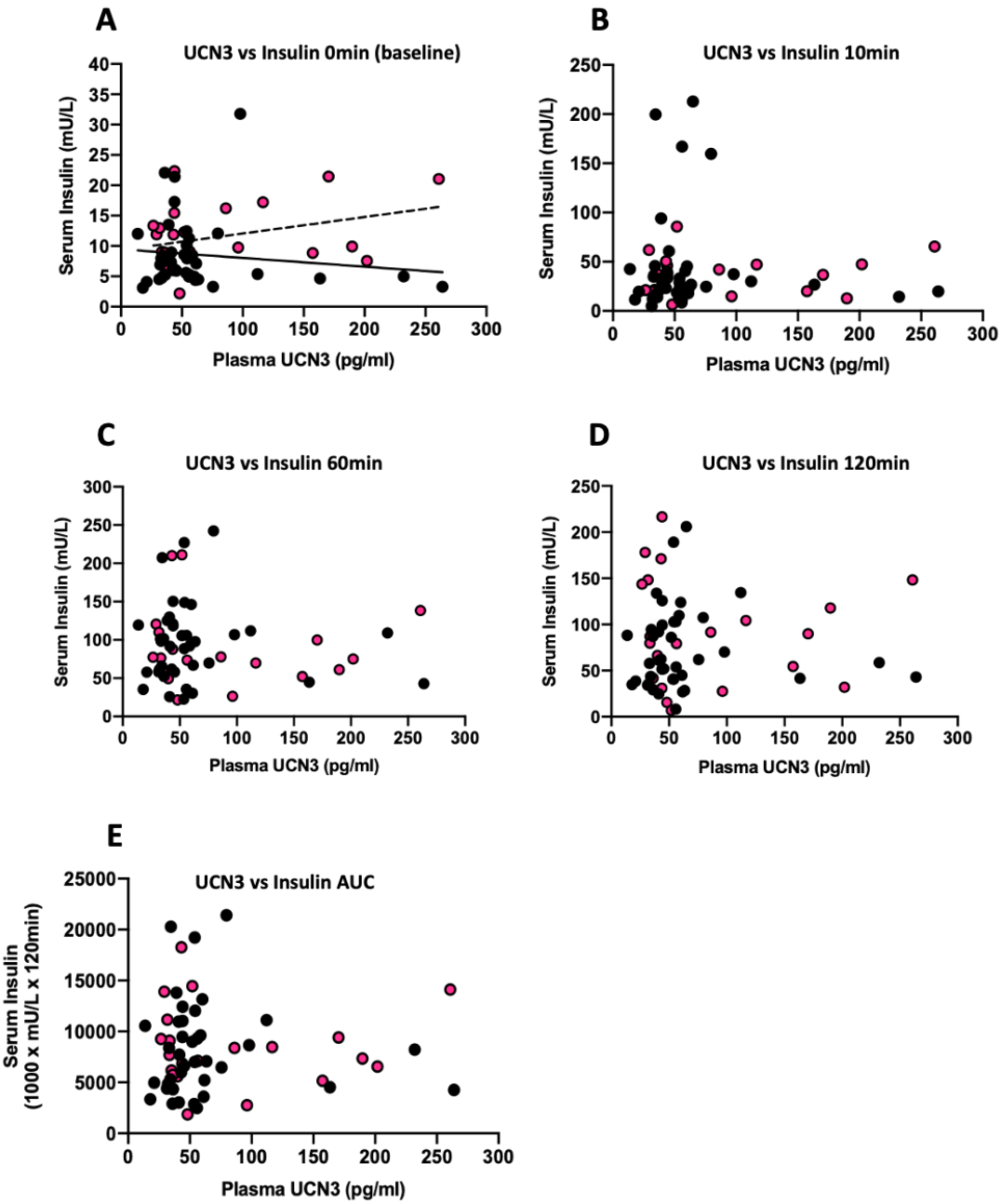

Figure 6-6 Relationship between UCN3 and serum insulin during an OGTT in pregnant women. Measurement of plasma UCN3 levels, fasted insulin (A) and insulin response to oral glucose in pregnant women during a routine 2-h 75 g OGTT between 26-34 weeks' gestation. Serum insulin measurements at 10 minutes (B), 60 minutes (C) and 120 minutes (D) following oral glucose with subsequent total insulin secreted during the course of the OGTT (E) are shown. Women diagnosed with GDM are represented by pink circles and women without GDM represented by black circles. Pearson's correlation coefficient was used for analysing correlation data. Dashed black line represents correlation coefficient line for women with GDM and solid black line for healthy women.

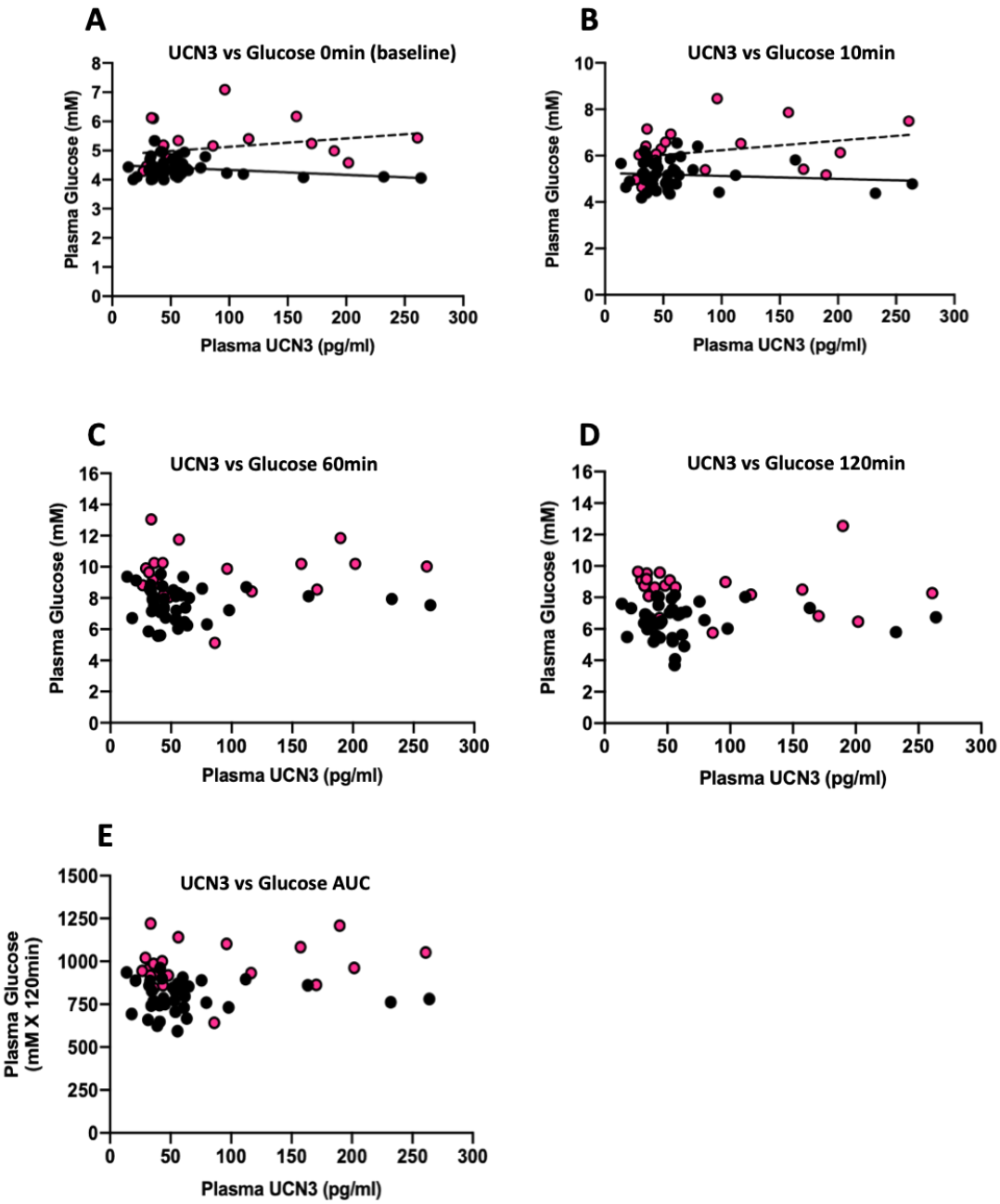

Figure 6-7 Relationship between UCN3 and plasma glucose during an OGTT in pregnant women. Measurement of plasma UCN3 levels, fasted plasma glucose (A) and glucose response during a routine 2-h 75 g OGTT in pregnant women between 26-34 weeks' gestation. Plasma glucose measurements at 10 minutes (B), 60 minutes (C) and 120 minutes (D) following oral glucose and glucose AUC (E) are shown. Women diagnosed with GDM are represented by pink circles and women without GDM represented by black circles. Pearson's correlation coefficient was used for analysing correlation data. Dashed black line represents correlation coefficient line for women with GDM and solid black line for healthy women.

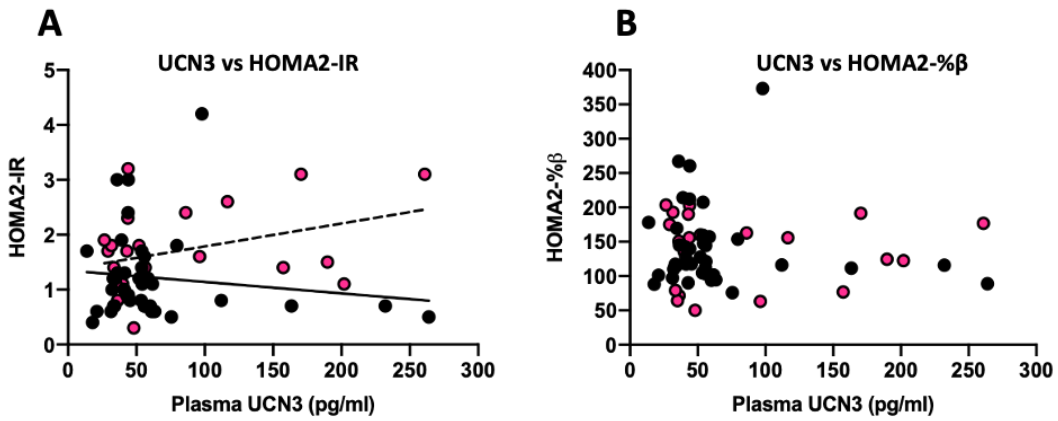

Figure 6-8 Relationship between UCN3 and HOMA2-IR and HOMA2-%β. HOMA2-IR (A) and HOMA2-%β (B) were calculated from insulin and glucose measurements obtained from pregnant women undergoing a routine 2-h 75 g OGTT between 26-34 weeks' gestation and compared to plasma UCN3 levels. Women diagnosed with GDM are represented by pink circles and women without GDM represented by black circles. Pearson's correlation coefficient was used for analysing correlation data. Dashed black line represents correlation coefficient line for women with GDM and solid black line for healthy women.

UCN3	All pregnant women (n=63)		Healthy women (n=40)		Women with GDM (n=23)	
	Pearson's R correlation coefficient	p value	Pearson's R correlation coefficient	p value	Pearson's R correlation coefficient	p value
Insulin correlations						
UCN3 vs. Insulin 0 min	0.096	0.456	-0.124	0.451	0.342	0.111
UCN3 vs. Insulin 10 min	-0.034	0.791	-0.075	0.646	0.177	0.419
UCN3 vs. Insulin 60 min	-0.062	0.640	-0.072	0.665	-0.008	0.971
UCN3 vs. Insulin 120 min	-0.004	0.976	-0.040	0.808	-0.011	0.961
UCN3 vs. Insulin AUC	-0.022	0.865	-0.062	0.711	0.044	0.846
Glucose correlations						
UCN3 vs. Glucose 0 min	0.153	0.230	-0.266	0.097	0.254	0.242
UCN3 vs. Glucose 10 min	0.187	0.143	-0.105	0.520	0.292	0.177
UCN3 vs. Glucose 60 min	0.178	0.174	0.030	0.854	0.140	0.546
UCN3 vs. Glucose 120 min	0.113	0.378	0.038	0.818	0.007	0.975
UCN3 vs. Glucose AUC	0.195	0.135	0.014	0.935	0.187	0.417
Homeostatic model assessment (HOMA) correlations						
UCN3 vs. HOMA2-IR	0.121	0.351	-0.134	0.423	0.365	0.087
UCN3 vs. HOMA2-%β	-0.057	0.661	-0.109	0.515	0.039	0.858

Table 6-3 Pearson's correlation coefficient data between UCN3 and insulin and glucose responses during OGTT in pregnant women. Correlations and statistical analysis for the entire cohort (All pregnant women) and women without (Healthy) and with GDM are shown.

6.3.4 Relationship between CRH and glucose tolerance in pregnant women with and without GDM

In the cohort of pregnant women sampled, Pearson's correlation coefficients were examined between plasma CRH and; 1) insulin response to oral glucose (Figure 6-9), 2) plasma glucose during OGTT (Figure 6-10) and 3) calculated surrogate indices for insulin resistance (HOMA2-IR) and β-cell function (HOMA2-%β) (Figure 6-11). Correlation coefficients were calculated for the entire cohort (i.e. all pregnant women both with and without GDM) as well as for women with and without GDM separately (Table 6-4).

No significant correlations between CRH and insulin responses were observed at any time point throughout the OGTT for the entire cohort (Figure 6-9). However, there was a weak positive correlation between CRH and basal glucose in the entire pregnant cohort, with a trend towards significance (r = 0.220; p =0.083) (Figure 6-10A). This correlation was similarly reflected by women with GDM independently (Table 6-4). At 10 minutes post glucose administration, the positive correlation between CRH and plasma glucose became statistically significant among the entire cohort (r = 0.306; p = 0.015) and also specifically in the GDM group (r = 0.502; p = 0.015) (Figure 6-10B & Table 6-4). No significant correlations between CRH and either HOMA2-IR or HOMA2-%β were observed (Figure 6-11).

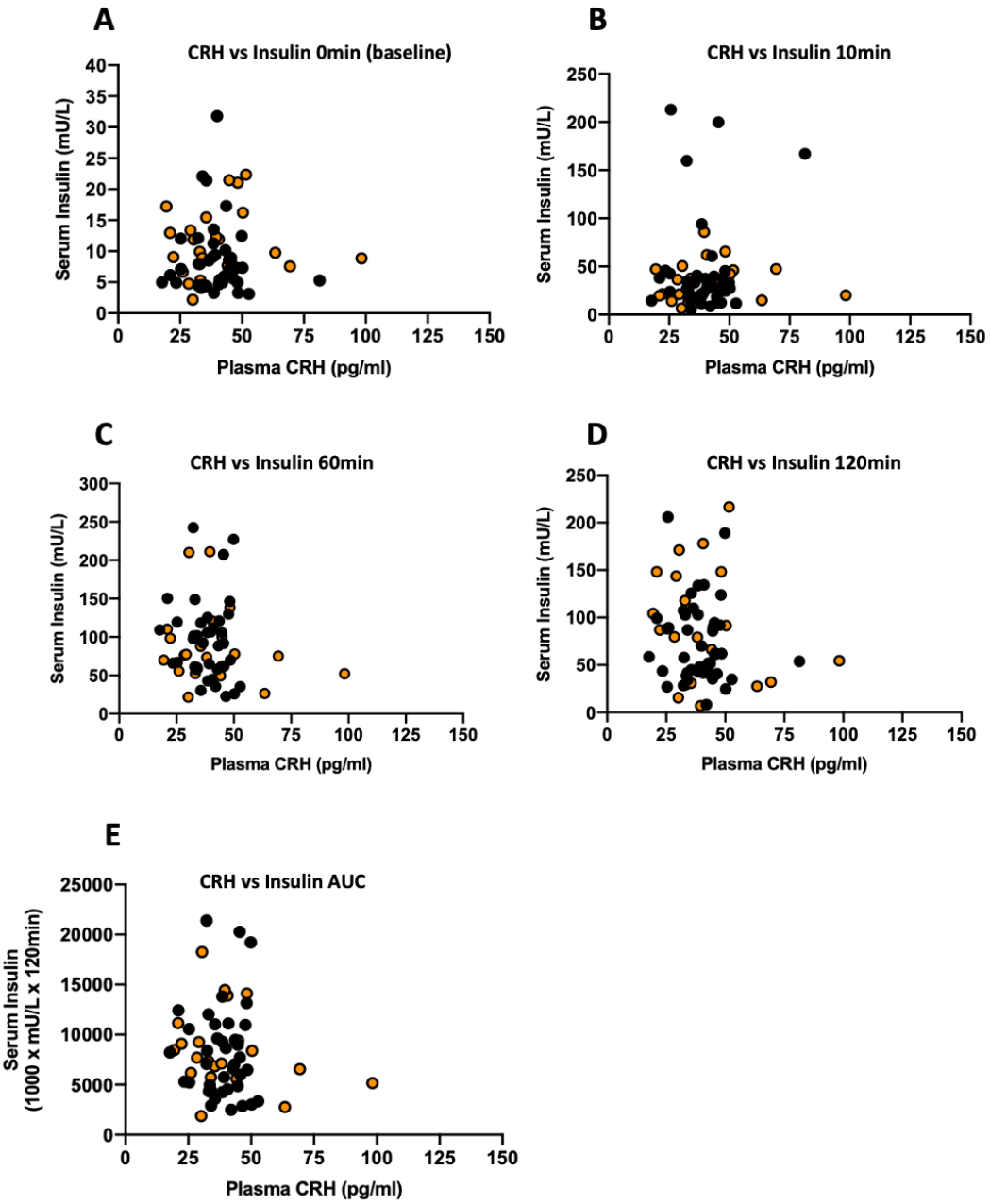

Figure 6-9 Relationship between CRH and serum insulin during an OGTT in pregnant women. Measurement of plasma CRH levels, fasted insulin (A) and insulin response to oral glucose in pregnant women during a routine 2-h 75 g OGTT between 26-34 weeks' gestation. Serum insulin measurements at 10 minutes (B), 60 minutes (C) and 120 minutes (D) following oral glucose with subsequent total insulin secreted during the course of the OGTT (E) are shown. Women diagnosed with GDM are represented by orange circles and women without GDM are represented by black circles. Pearson's correlation coefficient was used for analysing correlation data. No correlations were observed.

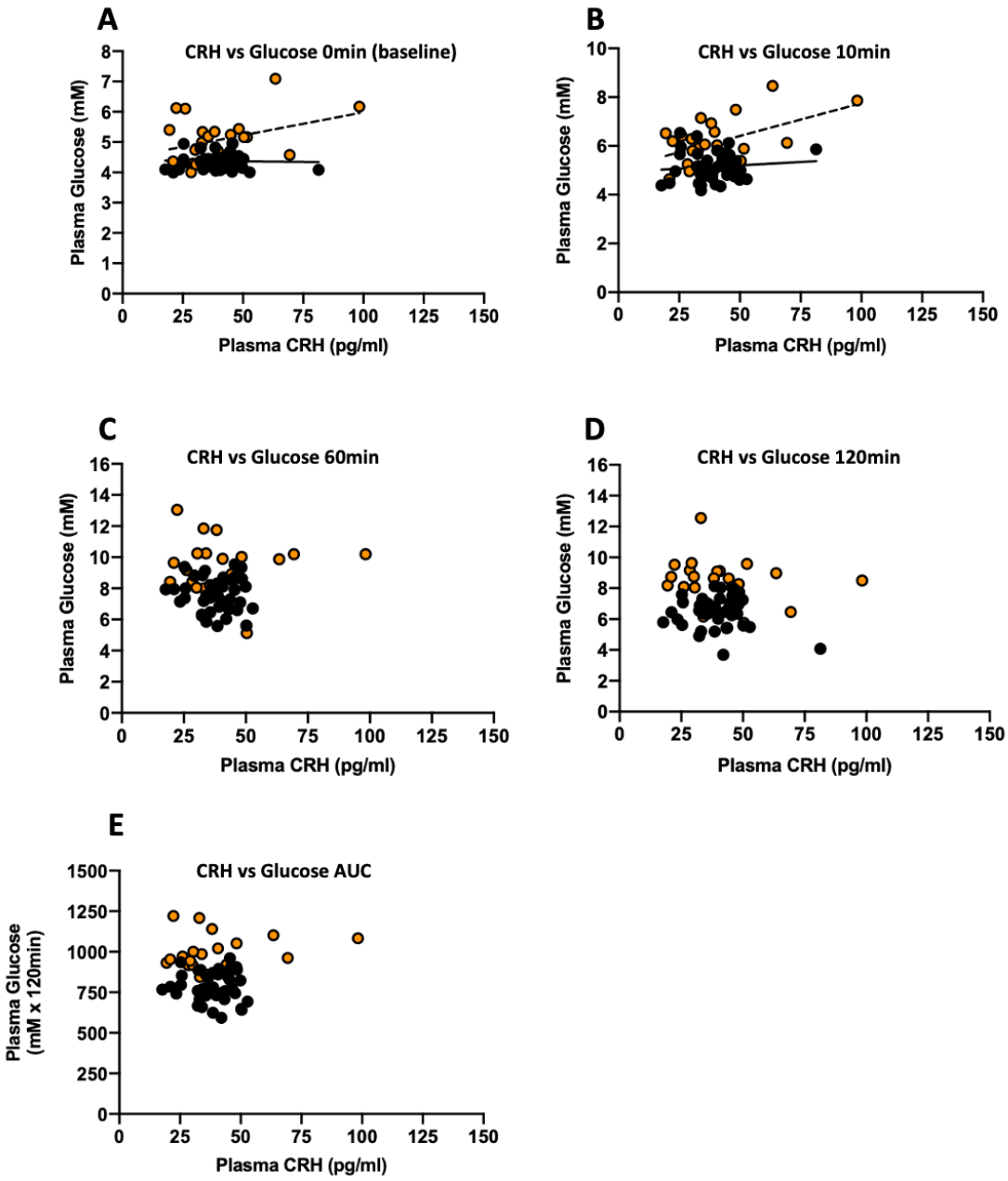

Figure 6-10 Relationship between CRH and plasma glucose during an OGTT in pregnant women. Measurement of plasma CRH levels, fasted plasma glucose (A) and glucose response during a routine 2-h 75 g OGTT in pregnant women between 26-34 weeks' gestation. Plasma glucose measurements at 10 minutes (B), 60 minutes (C) and 120 minutes (D) following oral glucose and glucose AUC (E) are shown. Women diagnosed with GDM are represented by orange circles and women without GDM are represented by black circles. Pearson's correlation coefficient was used for analysing correlation data. Dashed black line represents correlation coefficient line for women with GDM and solid black line for healthy women.

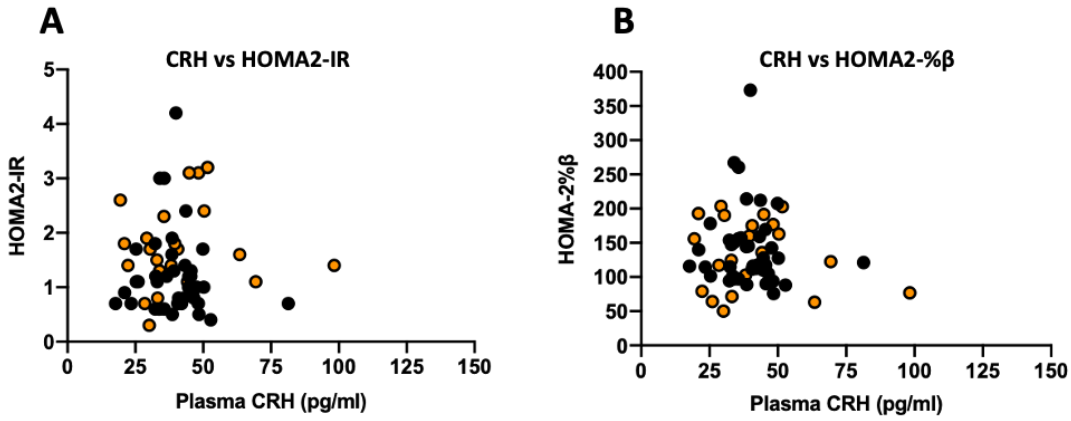

Figure 6-11 Relationship between CRH and HOMA2-IR and HOMA2-%β. HOMA2-IR (A) and HOMA2-%β (B) were calculated from insulin and glucose measurements obtained from pregnant women undergoing a routine 2-h 75 g OGTT between 26-34 weeks' gestation and compared to plasma CRH levels. Women diagnosed with GDM are represented by orange circles and women without GDM are represented by black circles. Pearson's correlation coefficient was used for analysing correlation data. No correlations were observed.

CRH	All pregnant women (n=63)		Healthy women (n=40)		Women with GDM (n=23)	
	Pearson's R correlation coefficient	p value	Pearson's R correlation coefficient	p value	Pearson's R correlation coefficient	p value
Insulin correlations						
CRH vs. Insulin 0 min	-0.003	0.979	-0.078	0.638	0.067	0.762
CRH vs. Insulin 10 min	0.098	0.444	0.143	0.378	0.076	0.729
CRH vs. Insulin 60 min	-0.132	0.314	-0.106	0.529	-0.170	0.451
CRH vs. Insulin 120 min	-0.142	0.266	-0.102	0.530	-0.191	0.383
CRH vs. Insulin AUC	-0.112	0.397	-0.022	0.897	-0.223	0.318
Glucose correlations						
CRH vs. Glucose 0 min	0.220	0.083	-0.030	0.854	0.358	0.094
CRH vs. Glucose 10 min	0.306	0.015*	0.102	0.533	0.502	0.015*
CRH vs. Glucose 60 min	0.028	0.834	-0.029	0.859	0.002	0.995
CRH vs. Glucose 120 min	-0.101	0.430	-0.146	0.369	-0.163	0.457
CRH vs. Glucose AUC	0.073	0.579	0.012	0.941	0.063	0.786
Homeostatic model assessment (HOMA) correlations						
CRH vs. HOMA2-IR	0.001	0.992	-0.097	0.558	0.089	0.685
CRH vs. HOMA2-%β	-0.105	0.419	-0.069	0.678	-0.160	0.465

Table 6-4 Pearson's correlation coefficient data between CRH and insulin and glucose responses during OGTT in pregnant women. Correlations and statistical analysis for the entire cohort (All pregnant women) and women without (Healthy) and with GDM are shown.

6.4 Discussion

Current understanding of the mechanisms of islet adaptations in pregnancy holds that circulating factors in maternal serum (particularly placental lactogens and pituitary derived prolactin) drive β-cell mass expansion and hyper-functionality. Our studies using pharmacological approaches in mice have uncovered a role for UCN2/CRHR2 signalling during pregnancy which contributes to the maintenance of maternal glucose tolerance by amplifying the insulin secretory response to glucose. Whilst most of the fundamental islet compensatory responses are similar between species, subtle differences exist between human and rodent pregnancy. This includes the cellular mechanisms responsible for β-cell mass expansion and may also extend to the signals regulating the overall β-cell adaptations. Therefore, the aim of the studies described in this chapter was to determine whether the signals we identified in rodent pregnancy are also involved in regulating human β-cell adaptations to pregnancy. A potential role for CRHR signalling in human islet adaptive mechanisms during pregnancy is supported by previous studies demonstrating the existence of the CRH and CRHR system in human pancreatic islets (Amisten et al., 2013; Schmid et al., 2011). Activation of CRH receptors *in vitro* can elevate insulin secretory responses in human islets, similar to the response we and others have demonstrated in rodent islets (Huising et al., 2010, Chapter 3). As our rodent data suggests that this mechanism may have physiological relevance within pregnancy, we used plasma samples from a cohort of pregnant women to investigate correlations between circulating CRH/UCN peptide levels and glucose tolerance in these women to address the hypothesis that placentally-derived CRH peptides are involved in β-cell adaptations during human pregnancy.

Although CRH has been studied extensively in human gestation (particularly its involvement in the timing of birth), very few studies have looked at maternal levels of its structurally related family members, the urocortins, during pregnancy. Pepels et al (2010) are the only other authors who have previously compared maternal plasma levels of all CRH peptides in pregnant and non-pregnant women. In this cross-sectional study (between 9 – 41 weeks' gestation) plasma levels of CRH peptides were evaluated in a total of 46 healthy pregnant women. The authors reported that UCN1 -2 and -3 levels did not show gestational age dependency changes in circulating levels, though all three peptide levels differed significantly from each other with mean UCN3 levels measured in maternal plasma being the highest. As expected, CRH was the only peptide to demonstrate a gestational age dependent exponential increase (Pepels et al., 2010). Assay of our pregnant cohort revealed that maternal circulatory levels of all urocortin peptides as well as CRH in the early third trimester were relatively low. However, similarly to Pepels et als' findings, maternal plasma levels of UCN3 were the highest amongst the urocortin peptides. We were unable to measure detectable levels of UCN1 in our samples (i.e. <15 pg/ml), consistent with the idea that levels of this peptide are not substantially elevated in pregnancy. The mean plasma level of

UCN1 during the third trimester in Pepels et als' study was ~22pg/ml. This is consistent with our samples also having very low levels of UCN1 which would require a more sensitive assay for measurement. Whereas we used ELISA's for peptide measurement, Pepels et al utilised radioimmunoassay methodologies which may have been more sensitive. However, our data regarding UCN2 and UCN3 plasma levels were consistent with previously reported values. Comparable levels of UCN2 were demonstrated between our studies (~15 pg/ml in both). We observed slightly higher mean plasma levels of UCN3 (62 pg/ml vs 32 pg/ml) and our CRH levels were approximately 20 times lower than those reported by Pepels et al (Pepels et al., 2010). CRH levels in the third trimester would have been expected to be much higher than what were measured in our cohort however, this discrepancy may be due in part to differences in how the samples were collected/processed.

We next grouped samples from women diagnosed with and without GDM and compared mean peptide levels. No significant differences in maternal plasma levels of CRH, UCN2 or UCN3 were measured between women with and without GDM. Given our *in vivo* data suggests that endogenous UCN2 facilitates the maintenance of maternal glucose tolerance in pregnancy (Chapter 4), it may have been expected that pregnancies complicated by GDM are associated with lower circulating levels of UCN2. However, as has been previously noted, just because a signal plays a role in islet adaptive responses to pregnancy does not necessarily mean its levels will correlate with GDM. As such, other key hormones implicated in modulating β-cell adaptations such as PL and PRL have been shown to be unaltered in women with GDM compared to healthy control women (Grigorakis et al., 2000; Retnakaran et al., 2016). Conversely, some studies have also reported higher maternal serum levels of PL and PRL are associated with reduced glucose tolerance in pregnancy and gestational diabetes (Ekinci et al., 2017; Henderson and Divon, 1998). These discrepancies highlight the difficulties of reproducibility and sensitivity of potential biomarkers when trying to translate experimental data to clinical application. With several hormonal parameters influencing the functional and morphological β-cell changes, it may be useful in future investigations to evaluate the clinical significance of the identified signals collectively. So far, there have been no specific biomarkers that have clearly demonstrated potential for clinical utility, although many show great promise including the more recently implicated hormones, kisspeptin and adiponectin. Both hormones are expressed and secreted by the placenta (J. Chen et al., 2006; Horikoshi et al., 2003b). Maternal kisspeptin levels rise throughout gestation whereas adiponectin levels decline with increasing gestation (Catalano et al., 2006; Dhillo et al., 2006; Horikoshi et al., 2003b; Lekva et al., 2017). The rise in circulating kisspeptin is now believed to be associated with facilitating the β-cell compensatory responses to pregnancy while the decline in adiponectin levels is commonly related to the changes in maternal insulin sensitivity across gestation though emerging evidence suggests adiponectin may also be involved in promoting maternal β-cell mass expansion through placental lactogen expression

(Bowe et al., 2019; Plows et al., 2018; Qiao et al., 2020, 2017; Retnakaran, 2017). Importantly, clinical studies have reported significantly lower circulating levels of kisspeptin and adiponectin in GDM pregnancies compared to normal healthy pregnancies (Bowe et al., 2019; Brink et al., 2016; J et al., 2014). These observations are consistent with both hormones playing a role in the development of GDM in humans and supports their use as potential biomarkers.

Other prospective biomarkers include the pro-inflammatory cytokine TNF-α and the satiety adipokine, leptin (Brink et al., 2016; Simpson et al., 2018). TNF-α and leptin are produced by both adipose tissue and the placenta though the latter is proposed to substantially contribute to the increased maternal levels of both hormones during pregnancy (J et al., 2014). Thus, levels of TNF-α have been demonstrated to be higher in late pregnancy compared to early pregnancy (Kirwan et al., 2002b; Rodrigo and Glastras, 2020). Similarly, circulating levels of leptin increase significantly across gestation (Lacroix et al., 2016; Misra and Trudeau, 2011). Both hormones are commonly associated with insulin sensitivity; TNF-α impairing insulin sensitivity and leptin enhancing insulin sensitivity, and therefore are presumed to play a role in the pathogenesis of GDM, where the physiological insulin resistance of normal pregnancy may be exaggerated in some women. Multiple studies have subsequently reported increased TNF-α levels in women with GDM compared to women without GDM (Brink et al., 2016; J et al., 2014) consistent with the theory that TNF-α may be an important mediator of the pathophysiological insulin resistance in GDM. Increased leptin levels have also been reported in GDM. In one study, elevated leptin concentrations, independent of adiposity, were positively correlated with an increased risk of GDM as early as 13 weeks (Qiu et al., 2004). The hyperleptinemia in GDM may be indicative of leptin resistance which is also a feature described in T2DM (Fischer et al., 2002). Though several biomarkers for GDM have been investigated, larger population studies are required to further validate such biomarkers before any clinical implementation.

Considering the mild phenotype we observed in our pregnant animal model (Chapter 4), this may suggest that the CRH system plays more of an ancillary role in maintaining islet adaptations. Therefore, any disturbances in this system may have minor effects in GDM progression that are difficult to detect in a human population as other mechanisms may compensate. It is also important to note that for UCN2 peptide measurements, the sample size (representing both GDM and non-GDM groups) was reduced owing to approximately half of sample values falling below the lower end of the assay sensitivity. Therefore, it is important that caution is taken when interpreting the results in this chapter and any observations should be regarded as preliminary until a larger number of samples are evaluated to confirm these initial findings.

The hormonal milieu of pregnancy can serve as an important determinant of pregnancy complications and therefore, relationships or associations between hormonal parameters and

pregnancy physiology may be able to provide evidence of the involvement of such biomolecules as well as potential indications of those at risk. The lack of any association between levels of CRH family ligands and GDM does not necessarily mean that these hormones do not play a role in maintaining pregnant glucose homeostasis, as discussed above. An analysis of correlations between these hormones and more detailed measures of glucose homeostasis across an OGTT could provide more insight into their potential roles in human pregnancy.

The correlations between plasma CRH, UCN2 and UCN3 peptides and OGTT responses in pregnant women with and without GDM provide evidence to suggest that UCN2 may have a similar physiological role in human pregnancy adaptations to that revealed in our mouse models. No correlation between UCN2 and basal fasted insulin was detected. However, a moderate-strength positive correlation (with a trend towards significance) between plasma UCN2 and glucose stimulated insulin responses at 60 min and over the duration of the OGTT (AUC) was observed. Despite these correlations not reaching statistical significance, power calculations suggest that a slightly higher GDM sample size (between 23 – 35) would have been required to show significance at the 0.05% level. Nevertheless, these data support that UCN2 does not initiate insulin secretion at low glucose but can amplify insulin secretion in response to glucose challenge without raising basal levels. This is consistent with our *in vitro* static insulin secretion data where a glucose-dependent potentiation of insulin secretion was shown by pancreatic islets (Chapter 3).

Our *in vivo* mouse model of pregnancy suggested that the primary mechanism of action of placental UCN2 was by directly influencing β-cell function as opposed to altering peripheral insulin sensitivity. Whether the same holds true in the human scenario was assessed in our pregnant cohort. Established methods for measuring insulin sensitivity and β-cell function *in vivo* are relatively complex hence larger scale clinical studies opt to perform the more straightforward oral glucose tolerance test. Fasting glucose and insulin measurements can then be used to estimate insulin resistance and β-cell function by applying the simple and reliable HOMA model. Therefore, HOMA2-IR and HOMA2-%β are commonly presented surrogate indices of these parameters with a higher readout for both indicative of greater insulin resistance and β-cell function respectively (Al-Mahmood et al., 2006; Imamura et al., 2013). Surprisingly, a weak positive correlation between UCN2 and plasma glucose was observed in the entire cohort and healthy women separately at 120 min. However, this was independent of any correlations detected between UCN2 and insulin response at the same time point or HOMA2-IR. This suggests that elevated circulating glucose levels may be as a result of high UCN2 activating other systems which drive glucose production, similar to the phenomenon seen in our acute UCN2 studies in lean and ob/ob mice (Chapter 5). However, limited conclusions can be made regarding the variable effect of UCN2 on glucose homeostasis as the exact mechanism of UCN2 influencing glucose production is unclear. Fundamentally, the minor association between UCN2 and overall

glucose parameters and the lack of correlation with HOMA2-IR suggests the positive correlation between UCN2 and insulin release is independent of changes in insulin sensitivity and is not secondary to higher circulatory glucose levels. The observation of a moderate-strength positive correlation between UCN2 and HOMA2-%β, is also consistent with the hypothesis from our mouse pregnancy studies that higher levels of maternal UCN2 can enhance islet β-cell function and thus potentiate glucose-induced insulin secretory responses. As with the correlations discussed above with UCN2 and OGTT insulin responses (at 60 min and AUC), the GDM sample size limited the statistical significance of the positive correlation between UCN2 and HOMA2-%β. Thus, increasing the sample size to 31 would have been needed for sufficient power, as determined by power calculations.

The positive correlations discussed above were generally specific to those women diagnosed with GDM, i.e. positive correlations between UCN2 and insulin responses were not present in women without GDM. A possible explanation for this observation could be that in normal healthy pregnancies, UCN2 may have a minor overall contribution to the functional β-cell adaptive responses as other signals compensate. Hence, only a mild phenotype is displayed with endogenous UCN2 blockade during pregnancy in our mouse model. However, women with GDM have impaired glucose homeostasis which may be as a result of insufficient β-cell adaptations and/or exaggerated insulin resistance. Inadequate or dysregulated release of placental-derived signals may be in part associated with these pathophysiological mechanisms. Therefore, their islets struggle to meet the maternal insulin demands. In this environment, the relatively modest effects of UCN2 to enhance β-cell secretory function may be important, as is evidenced by our correlations in this group of women. In fact, many of the correlations which were observed among the peptides in this study paralleled this phenomenon of correlations being detected specifically in GDM pregnancies. This could be an important consideration when evaluating the potential clinical applicability of CRH peptides but warrants further investigation in a larger GDM cohort.

UCN3 is also a selective ligand for CRHR2 (Lewis et al., 2001b) and, given the increasing evidence demonstrating the involvement of UCN3 in numerous metabolic pathways including mechanisms of insulin secretion and glucose homeostasis (Kuperman and Chen, 2008; Li et al., 2007), it is possible that placental UCN3 could have similar effects to those of UCN2 in islet adaptive responses to pregnancy. However, no correlations between plasma UCN3 and insulin responses to glucose or HOMA2-%β were observed suggesting that UCN3 most likely doesn't have a similar effect on the islets as UCN2, at least in human pregnancy, and further supports our suggestion that UCN2 is the endogenous ligand mediating the effects seen *in vivo* (Chapter 4). We observed an unexpected positive correlation between UCN3 and HOMA2-IR in women with GDM, suggesting that the peptide may be associated with higher degrees of insulin resistance. Also consistent with this suggestion is the positive correlation between UCN3 and fasted glucose

levels in women with GDM which taken together with the positive correlation between the peptide and fasted insulin may be indicative of greater insulin resistance. Although, it is important to note that an inverse of this relationship was observed in healthy women which would propose that higher levels of UCN3 is associated with reduced circulating glucose, possibly due to greater insulin sensitivity. Modulating insulin sensitivity has been proposed as another mechanism for UCN3 to influence glucose homeostasis. Conflicting observations have been reported in the literature however, with *in vitro* studies implying UCN3 can enhance glucose disposal and insulin signalling in rat skeletal muscle (Roustit et al., 2014), whereas animal models have either revealed no differences in insulin sensitivity between transgenic $UCN3^{+}$ mice and controls (Jamieson et al., 2011) or enhanced insulin sensitivity exhibited by UCN3 null mice (Li et al., 2007). Our human correlation data also appears to reveal variable effects of the peptide on glucose homeostasis, most likely via insulin signalling pathways, but it is evident that these are distinct from the mechanisms of its close family member, UCN2. Although there are very few clinical studies regarding UCN3 and metabolic syndromes, a recently published study exploring UCN3 and the associated risk of T2DM revealed increased levels of UCN3 are associated with unfavourable metabolic profiles in T2DM, including positive correlations with fasting blood glucose and insulin resistance (modelled by HOMA-IR) (Alarslan et al., 2020). Given that a potential pathophysiological feature of GDM is exaggerated insulin resistance, it is possible that UCN3 may be involved in the development of insulin resistance during pregnancy as it has been shown for T2DM. However, although mean plasma levels of UCN3 were slightly higher in women with GDM, we failed to observe any significant associations in comparison to healthy control women. Nevertheless, the correlations identified between UCN3 and insulin resistance most likely reflect the peptides actions at insulin target tissues as opposed to directly signalling via the pancreatic β-cell.

Whilst many features of rodent pregnancy are similar to humans, CRH and CRH binding protein (CRH-BP) are characteristic features in the physiology of human pregnancy (Behan et al., 1995; Robinson et al., 1989). Evidence in humans has now led to the widely accepted view that CRH is involved in the timing of birth by modulating signalling systems that control the contractile properties of the myometrium (Thomson, 2013). CRH-BP was originally isolated from human plasma and has since been detected in human placenta where it is thought to modulate the action and bioavailability of maternal CRH (Behan et al., 1995; Orth and Mount, 1987). In rodents, CRH-BP is primarily expressed in neuronal tissue however, CRH-BP transcripts and protein have been detected in rat adrenals (Chatzaki et al., 2002), though its peripheral role is still unclear. In humans the maternal circulatory pattern of CRH displays an exponential increase from the end of the first trimester, peaking at term and thus acts as a measure of the length of gestation (Campbell et al., 1987; McLean et al., 1995; Sasaki et al., 1988, 1987). This CRH secretory pattern is believed to be absent in rodents as earlier studies failed to detect CRH in the placenta of other

species including rats and guinea pig (Power and Schulkin, 2006; Robinson et al., 1989). However, we and others have since detected mRNA expression of CRH in mouse placenta and have measured low concentrations of the peptide during mouse pregnancy (Drynda et al., 2018, Chapter 4). Although the circulating profile of CRH and the results from our pharmacological studies in pregnancy suggest that CRH does not appear to play a role in the islet adaptation to mouse pregnancy, this does not necessarily mean that it does not play an important role in humans, particularly given the subtle differences in the physiology of pregnancy between both species.
Aside from its role in regulating parturition, it has also been proposed that placental CRH functions to alter maternal metabolism to stimulate maternal cortisol production and thus increase circulatory levels of glucose in the maternal bloodstream available for fetal consumption (Gangestad et al., 2012). Our observations from this study support this concept as a positive correlation between plasma CRH and basal glucose (with a trend towards significance) was displayed by all pregnant women. This correlation was maintained and reached significance at 10 minutes following glucose challenge, with a stronger correlation in women with GDM than healthy women. A possible explanation for the stronger correlation in GDM may be because women with GDM have a greater stress response compared to normal pregnancies. Thus, women with GDM have been shown to exhibit higher levels of biological markers of stress such as adrenaline, noradrenaline and cortisol (Feng et al., 2020), hormones which drive glucose mobilisation. Although there was no significant difference in mean plasma CRH levels between healthy women and women with GDM, those with GDM may be more sensitive to HPA axis activation and thus higher concentrations of CRH may result in higher plasma glucose levels. As no correlations between CRH and plasma insulin, HOMA2-IR or HOMA2-%β were detected, the relationship between CRH and glucose most likely reflects indirect effects of CRH on glucose homeostasis via cortisol-mediated glucose mobilisation and independent of any direct effects either on the islets or insulin target tissues.

It is evident from the literature and our studies thus far, that the CRH system plays several roles in both mouse and human pregnancy physiology, involving receptors in and outside of the HPA axis. Importantly, our data is consistent with a conserved role for endogenous UCN2 in both species, directly regulating the physiological islet adaptive mechanisms to support the insulin demands of pregnancy. The data shown here in pregnant women requires further validation but is the first piece of evidence to support a role for endogenous UCN2 in fine-tuning the compensatory β-cell adaptations to maternal insulin resistance during human pregnancy. Our results potentially suggest that CRH and UCN3 may also subtly influence glucose homeostasis during pregnancy, although these are most likely through effects on the HPA axis and insulin target tissues respectively rather than direct effects on the pancreatic islets. Deciphering the interplay between these different signals will lead to a more comprehensive understanding of the pathophysiology of gestational diabetes and may offer novel diagnostic or therapeutic strategies.

Supplementary patient characteristics

	All Participants	Healthy	GDM
No. of Participants	63	40	23
Age (Mean±SE)	33.3±0.72	33.55±0.92	32.92±1.16
BMI	30.76±0.87	29.36±1.09	33.30±1.33*
Gestation (weeks) (range)	29.27±0.18 (26-33)	29.30±0.19 (26-33)	29.22±0.31 (26-33)
Ethnicity (%) Asian Black White Other	 6.35% 19.05% 46.03% 28.57%	 5% 20% 47.5% 27.5%	 8.70% 17.39% 43.48% 30.44%

* p<0.05 GDM vs Healthy; T-test

Table 6-5 Characteristics of the pregnant women.

Chapter 7

Chapter 7 General Discussion

7.1 Introduction

Diabetes is a global health burden, affecting approximately 460 million people worldwide (Saeedi et al., 2019). Of the three main types of diabetes, an upsurge in T2DM and GDM are particularly contributing to the overall rise in diabetes prevalence (Ferrara, 2007; Saeedi et al., 2019). Gestational diabetes currently affects ~7% of pregnancies worldwide (Behboudi-Gandevani et al., 2019) and poses a serious public health concern. There is a growing need to mitigate the associated acute adverse maternal and fetal complications of the disease and subsequently reduce the future metabolic health risks for both mother and child (Reece, 2010).

Despite GDM being a common pregnancy complication, a great deal is still unknown about the physiological mechanisms believed to be at the epicentre of the disease pathophysiology which includes the compensatory pancreatic islet adaptations (Baeyens et al., 2016; Ernst et al., 2011; Moyce and Dolinsky, 2018; Pasek and Gannon, 2013). Normally, the physiological increase in maternal insulin resistance during pregnancy is countered by coordinated pancreatic β-cell adaptations (i.e. β-cell mass expansion and increased glucose-stimulated insulin secretion) to match the insulin requirements needed to maintain maternal glucose homeostasis (Moyce and Dolinsky, 2018; Rieck and Kaestner, 2010; Sorenson and Brelje, 1997). However, these normal metabolic adaptations to pregnancy do not adequately occur in all pregnancies and insufficient β-cell adaptative mechanisms driven by potential underlying β-cell defects which are exacerbated by the pregnancy associated insulin resistance, results in maternal hyperglycaemia and GDM. The fact that the acquired insulin resistance during pregnancy is not the same in all individuals and women with GDM exhibit lower insulin responses to glucose compared with pregnant women without GDM, suggests that some individuals may in fact be more susceptible to the inability to mount a robust β-cell response during pregnancy (Buchanan, 2001). Moreover, there is also the view that placental dysfunction and alterations in secreted signals by the placental stimulating the β-cell adaptations may also be involved in the pathophysiological mechanisms of GDM (Berberoglu, 2019). Research efforts so far have uncovered the contribution of several placental and non-placental signals influencing islet responses to pregnancy (Bowe et al., 2019; Drynda et al., 2015; Sorenson and Brelje, 2009, 1997). However, the general consensus currently stands that there are most likely a plethora of unidentified signals which may also play a role in these β-cell adaptations. This hypothesis is further supported by observations showing that inhibition of the currently identified signals such as the lactogenic hormones or kisspeptin do not completely terminate β-cell adaptive responses (Banerjee et al., 2016b), suggesting other mediators are involved.

Recent attempts to identify putative pathways for placental crosstalk with the islet in mice have revealed approximately 80 GPCR ligands expressed in the placental secretome for which the islets express the corresponding receptors, highlighting several potential interactions between placenta and islet which could be involved in pregnancy-induced islet adaptations (Drynda et al., 2018). One such network identified by these studies was the hypothalamic neuroendocrine CRH system which prompted the investigations presented in this thesis.

7.2 Overview of findings and key considerations

The difficulty in obtaining tissue samples from pregnant women with and without GDM coupled with the complex demands of generating animal models that fully recapitulate the disease, make studying the molecular mechanisms and treatment options for GDM challenging (Pasek and Gannon, 2013). Nevertheless, *in vitro* and *in vivo* approaches have allowed for the study of novel signals involved in the pancreatic islet adaptations to pregnancy. This project tested a series of hypotheses, using isolated mouse islets, pharmacological manipulation in mice and samples from a clinical cohort of pregnant women to investigate the potential role (s) of CRH and CRH-like peptides (i.e. urocortins) in the maternal pancreatic islet adaptations to pregnancy.

The results presented in chapter 3 confirmed a CRH receptor (CRHR) system is present in mouse islets and is capable of influencing islet function, as demonstrated by the potentiation of glucose-stimulated insulin secretion (GSIS) following either CRHR1 or CRHR2 activation. Though the static incubation studies provided variable data on the effects of islet CRHR activation on insulin secretion, these initial experiments revealed first indications that male and female islets may have characteristic responses to different CRHR ligands and cognate receptor activation. As such, male islets appeared more effective at potentiating GSIS via CRHR1 and CRH whereas in female islets CRHR2 and selective agonists against this receptor seemed to be more involved. However, subsequent, dynamic perifusion studies were able to consistently show that in female islets, activation of both CRHR subtypes could significantly enhance insulin secretion in a glucose dependent manner. Though limited studies have commented on the physiological relevance of the effects of CRH/UCN on islet function, our results from chapter 3 hinted at the fact that although both CRHRs may have similar roles, perhaps under different physiological scenarios, differential receptor activation occurs to enhance β-cell function.

The next logical step was to investigate the potential physiological relevance of the CRH system in pregnancy. This was prompted by previous studies within the group which had demonstrated mRNA expression levels of CRH ligands are altered during pregnancy in the placenta (Drynda et al., 2018). Characterisation of islet CRHR and placental and circulating CRH ligands during mouse pregnancy in Chapter 4, revealed an intriguing shift in CRH receptor ratio (i.e. a decrease

in CRHR1) during pregnancy as well as a significant increase in circulating maternal UCN2 despite all CRH-like peptides being expressed by the mouse placenta. These findings underpinned the first pieces of evidence demonstrating that CRH-like peptides and receptors are influenced by the pregnancy environment, suggesting a physiological role for CRHR signalling in modifying islet function or morphology. Subsequent studies in this chapter investigated the physiological consequence of pharmacologically blocking endogenous CRH/UCN-peptides on β-cell adaptations and glucose homeostasis during mouse pregnancy. For these experiments, osmotic minipumps containing non-selective or selective CRHR antagonists were subcutaneously implanted in pregnant or non-pregnant mice and subsequent metabolic phenotype assessed. The *in vivo* studies from chapter 4 thus revealed a pregnancy- and receptor- specific phenotype with pregnant mice exhibiting a mild and transient impairment to glucose tolerance following non-selective CRHR blockade which was associated with a decrease in glucose-stimulated plasma insulin levels, but not basal fasted insulin. A similar impairment to glucose tolerance was exhibited with selective CRHR2 blockade but was not observed with CRHR1 blockade, nor in non-pregnant mice. Consistent with the rise in circulating UCN2, these results support that UCN2 is the endogenous ligand involved in maintaining glucose homeostasis specifically during pregnancy. Although the placenta is the most likely source of the increase in maternal circulating UCN2 (given the high placental peptide expression and equivalent lower circulating levels in males (data not shown) and non-pregnant females), the skeletal muscle and skin (where UCN2 is also highly expressed) (Chen et al., 2004) could arguably represent an alternative source. It is possible that under the influence of a separate placental signal, these and other peripheral tissues could be influenced to synthesise and secrete UCN2 throughout gestation. Irrespective of the source of UCN2, the core conclusion still remains that during pregnancy, increased maternal UCN2 is involved in supporting β-cell endocrine capacity.

Interestingly, the effects of endogenous UCN2 appear to be confined to amplifying the β-cell insulin secretory response. The possibility of other off-target effects cannot be completely ruled out or whether the effects of UCN2 could potentially involve a more integrative islet-response than simply a direct effect on the β-cells given other pancreatic islet cells (i.e. somatostatin secreting δ-cells) have also been reported to express CRHR2 (van der Meulen et al., 2015). These considerations are intriguing but beyond the scope of this project. However, the data are consistent with; 1) the effect of UCN2 on insulin release following direct activation of CRHR2, 2) the lack of detectable changes to insulin sensitivity *in vivo*, 3) the decrease in glucose stimulated plasma insulin with antagonist treatment *in vivo* - albeit non-significant and variable, 4) and the absence of any changes to β-cell proliferation. These observations would therefore suggest that UCN2 directly signals via its cognate receptor on β-cells with the overall effect of potentiating insulin secretion. A next step in further understanding the physiological mechanisms by which UCN2 influences pregnant islet function might be to generate a β-cell- specific CRHR2 knockout mouse

and/or a placenta-specific UCN2 knockout mouse. These could then be utilised for *in vivo* studies to definitively assess whether the mediator is of placental origin and the effects observed are directly β-cell mediated. This type of model may thus allow further confirmatory and explorative studies into the mechanisms of UCN2 on islet adaptive responses. Generating novel genetically modified mouse strains is time-consuming and costly, with characterisation of the phenotype required to ensure tissue specificity. This type of model was not justifiable or appropriate prior to the initial *in vitro* and pharmacological *in vivo* approaches contained in this thesis which have formed the necessary groundwork to address the primary hypothesis.

Having found that endogenous placental UCN2 has a beneficial effect on islet function during pregnancy, the studies in chapter 5 used a reverse approach. Exogenous UCN2 was administered via osmotic minipumps to investigate whether the beneficial insulinotropic effects of UCN2 could be replicated in another model of impaired glucose homeostasis - the insulin resistant obese ob/ob mouse model. Overall, these experiments highlighted the variable effect of UCN2 on glucose homeostasis between acute and chronic administration of the peptide and the challenges in replicating the pregnancy associated UCN2 profile as a potential therapeutic intervention. Depending on the peptide dosing, mice administered UCN2 either exhibited a significant rise in blood glucose levels or no significant alterations to overall glucose homeostasis. Though we were unable to investigate the underlying mechanisms responsible for these observations, it is most likely that supraphysiological doses of UCN2 may activate the sympathetic nervous system and/or the adrenal gland directly to stimulate catecholamine (i.e. adrenaline and noradrenaline) release which drive glucose mobilisation. Therefore, the significant rise in blood glucose levels do not necessarily represent the physiological function of chronic UCN2 throughout pregnancy. An endogenous pregnancy signal having beneficial effects on islet function does not necessarily guarantee that this peptide will have similar effects outside of pregnancy when given exogenously. The lack of effect of chronic exogenous UCN2 on overall glucose homeostasis in another animal model of impaired glucose homeostasis suggests that something specific about the pregnancy environment enables UCN2 to facilitate the pancreatic islet adaptations only during gestation. For example, it could be that UCN2 works in synergy with other placental signals or additional endocrine systems during pregnancy and therefore, individually, the peptide may not demonstrate positive effects on glycaemic control. This again highlights the complexity of the signals which may be involved in the compensatory islet adaptations and the importance of understanding the interplay between the placenta and the islet. Alternatively, an additional factor to consider with the studies in this chapter is the choice of animal model. Perhaps the insulin resistance in ob/ob mice is more severe than that which would be present in pregnancy and therefore the mild effects of UCN2 is unable to improve glycaemic control. However, due to the limitations in biological replicates included within these particular experiments, further validation would be necessary. It would also be interesting to use an alternative animal model of a milder

metabolic phenotype possibly induced by high-fat diet to assess whether the limited therapeutic potential of UCN2 still stands.

Nevertheless, signalling molecules identified to have influential roles in critical pathways of pregnant islet responses can also be extremely valuable as candidates for clinical translation and may potentially serve as predictive markers to indicate those at risk of GDM. Therefore, the studies presented in chapter 6 investigated the translational relevance of our identification of UCN2/CRHR2 signalling as a novel mechanism to facilitate enhanced glucose-induced insulin secretion during pregnancy in human clinical samples. Plasma levels of CRH-like peptides were measured and analysed for correlations between various glycaemic parameters measured during a routine oral glucose tolerance test in pregnant women. Despite no significant differences in UCN2, UCN3 or CRH peptide levels between healthy (no GDM) and women with GDM, our preliminary findings did suggest that UCN2 was positively associated with insulin responses to glucose challenge and the surrogate index of β-cell function, HOMA2-%β. However, these were only noticeable trends and did not reach statistical significance. Nonetheless, these observations are consistent with the theory that higher levels of maternal UCN2 can directly enhance islet β-cell function and suggest a conserved mechanism between mouse and humans. Conversely, although additional trends were observed with UCN3, CRH and indicators of insulin sensitivity and circulating glucose levels, respectively, these likely reflected indirect effects of the peptides on glucose homeostasis and did not parallel the positive associations that had been observed by UCN2. Future work would initially focus on expanding these studies as the data suggested some very interesting correlations that could potentially reflect the effects of different members of the CRH family on different aspects of glucose metabolism and homeostasis during pregnancy. Many of the intriguing associations observed in the clinical data were specifically apparent in women with GDM and increasing the size of the GDM cohort would also be valuable to confirm the trends seen.

In summary, the studies conducted throughout this project have been valuable in elucidating a novel role for a placental-islet UCN2 system, contributing to the islet adaptive responses to pregnancy as illustrated in Figure 7-1. The mechanisms mediating the responses appear to be receptor-specific and the data are consistent with the endogenous signalling ligand most likely to be UCN2, supporting the β-cell adaptations by amplifying the insulin secretory response to the metabolic demand. Moreover, these mechanisms appear to be unique to pregnancy and there is plausible evidence to suggest these mechanisms are conserved between mouse and humans.

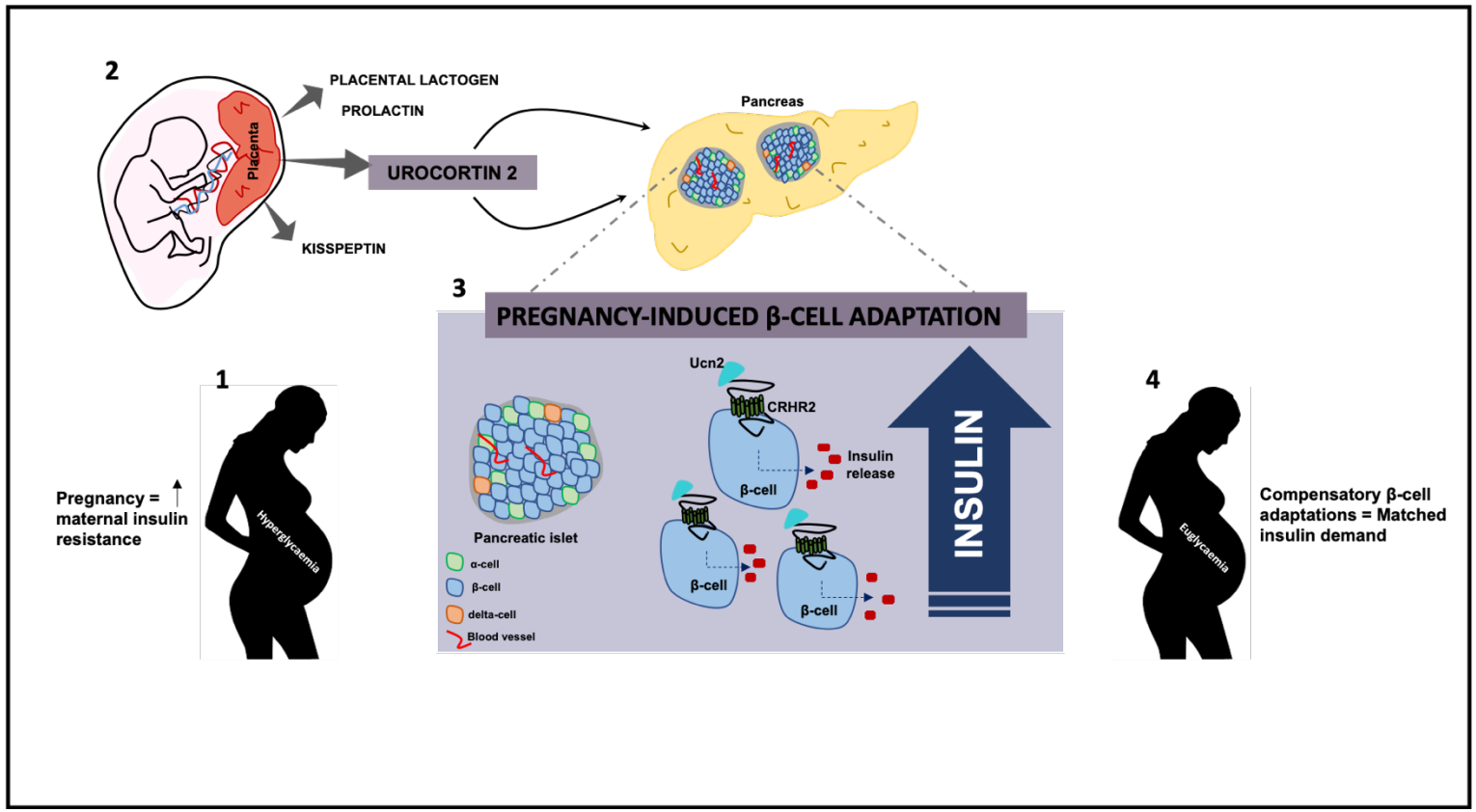

Figure 7-1 Schematic diagram illustrating the possible functional effects of placental UCN2 and β-cell CRHR2 on pregnancy-induced islet adaptive responses. 1) Physiological increase in maternal insulin resistance 2) Placental secretion of various biological mediators into maternal circulation to counter the insulin resistance 3) UCN2 signals via β-cell type 2 CRH receptors to enhance insulin output to meet the maternal insulin requirements 4) Maternal euglycaemia is maintained.

7.3 Future translational perspectives

As discussed in chapter 1 (section 1.4.3), management of GDM largely focuses on intensive monitoring of maternal glucose levels with medical nutrition therapy and the intervention of pharmacotherapies (i.e. insulin or Metformin), if glycaemic targets are not met. As was illustrated in chapter 3, activation of CRHR2 by exogenous UCN2 amplified the insulin secretory response under conditions of elevated glucose suggesting the peptide has the capacity to modulate the endocrine response of the β-cell. This raises the possibility that the insulinotropic effects of UCN2 could be harnessed therapeutically as seen with other insulinotropic GPCR therapies such as GLP-1 analogues. However, the translational value of UCN2 as a potential therapeutic target is difficult to assess at this point.

Unlike other placental factors implicated in pregnancy-induced islet adaptive responses, the mechanisms of UCN2 appeared to be confined to direct actions on β-cell insulin release without influencing overall β-cell mass. The physiological significance of this difference in mode of action is uncertain but there may be therapeutic advantages in the ability of UCN2 to specifically enhance GSIS without the clinical challenges of manipulating β-cell proliferation. Unfortunately, our studies in ob/ob mice (chapter 5), where we tried to mimic the beneficial pregnancy effects of UCN2 to positively influence glycaemic control suggest that the therapeutic efficacy of UCN2 treatment may be limited. Determining the correct dosing and time course of exogenous UCN2 would be a critical challenge in determining any beneficial versus undesirable effects on glucose homeostasis. The finding that UCN2 is capable of inducing large glucose excursions in itself limits its likely use as a therapy, though with further study it is possible it could be used in combination with other identified mediators of islet adaptations.

The data provided in this thesis does however suggest that UCN2 could potentially be of clinical value in screening strategies for assessing GDM risk. Currently, no specific biomarkers in the screening of GDM risk have demonstrated clinical value, with most proposed markers lacking sensitivity, specificity and reproducibility. The identification of novel biomarkers could allow for those at risk of GDM to be identified earlier (in comparison to the third trimester OGTT), enabling earlier intervention strategies and thus reduce adverse maternal and fetal consequences as a result of hyperglycaemic exposure. Although preliminary, our clinical data (chapter 6) suggests that UCN2 could represent a potential biomarker for assessing GDM risk and warrants further investigation. Consistent with our *in vitro* and *in vivo* observations, UCN2 was positively associated with insulin responses to oral glucose and HOMA2-%β suggesting that women with lower circulatory levels may represent a subset of patients at risk of impaired compensatory islet

adaptations and possibly susceptible to GDM. However, no significant overall difference was demonstrated between healthy women and women with GDM possibly suggesting that on its own, UCN2 may not exhibit the sensitivity that is required. Therefore, the candidacy of UCN2 as a biomarker for GDM will most likely be ideal in a combination or panel of other robust biomarkers to overcome the lack of sensitivity and specificity and may enable more subtle metabolic perturbations to be identified in early pregnancy. Of course, the proposition of UCN2 as part of any potential panel would require further validation in a larger cohort but with the prospect of integrating several predictive biomarkers in current clinical risk prediction models, the applicability of UCN2 may be more promising.

7.4 Concluding remarks

There is a growing research effort to identify placental signals involved in mediating the compensatory β-cell adaptations to pregnancy. Understanding these signals and potential mechanisms may not only elucidate more understanding of the pathophysiology of GDM, offering avenues for therapeutic intervention for this pregnancy complication, but also extends possible strategies to the broader context of diabetes. This thesis has identified a novel role for CRHR2 signalling in the β-cell adaptive responses to pregnancy in the mouse with endogenous placental UCN2 being the likely signal mediating this adaptation. Blocking an endogenous CRHR2 ligand during gestation induces a mild and transient glucose intolerance suggesting that CRHR2 signalling contributes to maintaining maternal normoglycaemia during pregnancy analogous with a mechanism of amplifying the insulin secretory response to the maternal insulin demand. Further investigations presented here in another animal model of impaired glucose homeostasis and in clinical studies appear to support the pregnancy-specificity of this signal which may be conserved in mouse and human gestation. The mild phenotype observed in response to CRHR2 blockade rather than overt gestational diabetes, along with subtle associations and trends in the clinical data, suggests that UCN2 may act in concert with other placental signals to fine-tune the compensatory β-cell adaptations. As yet, unidentified signals may also play a significant role in the communication between the placenta and pancreatic β-cells and deciphering the interplay between these different signals will lead to continued future advancements within the field.

References

ADA, A.D., 2019. 2. Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes—2019. Diabetes Care 42, S13–S28. https://doi.org/10.2337/dc19-S002

Adão, R., Santos-Ribeiro, D., Rademaker, M.T., Leite-Moreira, A.F., Brás-Silva, C., 2015. Urocortin 2 in cardiovascular health and disease. Drug Discov. Today 20, 906–914. https://doi.org/10.1016/j.drudis.2015.02.012

Ahrén, B., 2009. Islet G protein-coupled receptors as potential targets for treatment of type 2 diabetes. Nat Rev Drug Discov 8, 369–385. https://doi.org/10.1038/nrd2782

Akpan, J.O., Gardner, R., Wagle, S.R., 1974. Studies on the effects of insulin and acetylcholine on activation of glycogen synthase and on glycogenesis in hepatocytes isolated from normal fed rats. Biochemical and Biophysical Research Communications 61, 222–229. https://doi.org/10.1016/0006-291X(74)90556-7

Alarslan, P., Kocabas, G.U., Demir, I., Guler, A., Bozkaya, G., Aslanipour, B., Calan, M., 2020. Increased urocortin 3 levels are associated with the risk of having type 2 diabetes mellitus. Journal of Diabetes 12, 474–482. https://doi.org/10.1111/1753-0407.13020

Alcazar, O., Buchwald, P., 2019. Concentration-Dependency and Time Profile of Insulin Secretion: Dynamic Perifusion Studies With Human and Murine Islets. Front. Endocrinol. 10. https://doi.org/10.3389/fendo.2019.00680

Alfadhli, E.M., 2015. Gestational diabetes mellitus. Saudi Med J 36, 399–406. https://doi.org/10.15537/smj.2015.4.10307

Al-Mahmood, A.K., Ismail, A.A.-S., Rashid, F.A., Wan Bebakar, W.M., 2006. Insulin Sensitivity and Secretory Status of a Healthy Malay Population. Malays J Med Sci 13, 37–44.

American Diabetes Association, A.D., 2015. 12. Management of Diabetes in Pregnancy. Diabetes Care 38, S77–S79. https://doi.org/10.2337/dc15-S015

Amisten, S., Atanes, P., Hawkes, R., Ruz-Maldonado, I., Liu, B., Parandeh, F., Zhao, M., Huang, G.C., Salehi, A., Persaud, S.J., 2017. A comparative analysis of human and mouse islet G-protein coupled receptor expression. Sci Rep 7. https://doi.org/10.1038/srep46600

Amisten, S., Salehi, A., Rorsman, P., Jones, P.M., Persaud, S.J., 2013. An atlas and functional analysis of G-protein coupled receptors in human islets of Langerhans. Pharmacol. Ther. 139, 359–391. https://doi.org/10.1016/j.pharmthera.2013.05.004

Ander, S.E., Diamond, M.S., Coyne, C.B., 2019. Immune responses at the maternal-fetal interface. Science Immunology 4. https://doi.org/10.1126/sciimmunol.aat6114

Andersen, M.D., Alstrup, A.K.O., Duvald, C.S., Mikkelsen, E.F.R., Vendelbo, M.H., Ovesen, P.G., Pedersen, M., 2018. Animal Models of Fetal Medicine and Obstetrics. Experimental Animal Models of Human Diseases - An Effective Therapeutic Strategy. https://doi.org/10.5772/intechopen.74038

Ashcroft, F.M., Gribble, F.M., 1998. Correlating structure and function in ATP-sensitive K+ channels. Trends in Neurosciences 21, 288–294. https://doi.org/10.1016/S0166-2236(98)01225-9

Association, A.D., 2019. 2. Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes—2019. Diabetes Care 42, S13–S28. https://doi.org/10.2337/dc19-S002

Atègbo, J.-M., Grissa, O., Yessoufou, A., Hichami, A., Dramane, K.L., Moutairou, K., Miled, A., Grissa, A., Jerbi, M., Tabka, Z., Khan, N.A., 2006. Modulation of adipokines and cytokines in gestational diabetes and macrosomia. J. Clin. Endocrinol. Metab. 91, 4137–4143. https://doi.org/10.1210/jc.2006-0980

Baeyens, L., Hindi, S., Sorenson, R.L., German, M.S., 2016. β-Cell Adaptation in Pregnancy. Diabetes Obes Metab 18, 63–70. https://doi.org/10.1111/dom.12716

Bagosi, Z., Csabafi, K., Balangó, B., Pintér, D., Szolomájer-Csikós, O., Bozsó, Z., Tóth, G., Telegdy, G., Szabó, G., 2018. Anxiolytic- and antidepressant-like actions of Urocortin 2 and its fragments in mice. Brain Research 1680, 62–68. https://doi.org/10.1016/j.brainres.2017.12.011

Baigent, S.M., Lowry, P.J., 2000. mRNA expression profiles for corticotrophin-releasing factor (CRF), urocortin, CRF receptors and CRF-binding protein in peripheral rat tissues. J. Mol. Endocrinol. 25, 43–52. https://doi.org/10.1677/jme.0.0250043

Bale, T.L., Anderson, K.R., Roberts, A.J., Lee, K.-F., Nagy, T.R., Vale, W.W., 2003. Corticotropin-releasing factor receptor-2-deficient mice display abnormal homeostatic

responses to challenges of increased dietary fat and cold. Endocrinology 144, 2580–2587. https://doi.org/10.1210/en.2002-0091

Banerjee, R.R., Cyphert, H.A., Walker, E.M., Chakravarthy, H., Peiris, H., Gu, X., Liu, Y., Conrad, E., Goodrich, L., Stein, R.W., Kim, S.K., 2016a. Gestational Diabetes Mellitus From Inactivation of Prolactin Receptor and MafB in Islet β-Cells. Diabetes 65, 2331–2341. https://doi.org/10.2337/db15-1527

Bao, W., Baecker, A., Song, Y., Kiely, M., Liu, S., Zhang, C., 2015. Adipokine levels during the first or early second trimester of pregnancy and subsequent risk of gestational diabetes mellitus: A systematic review. Metabolism - Clinical and Experimental 64, 756–764. https://doi.org/10.1016/j.metabol.2015.01.013

Barbour, L.A., McCurdy, C.E., Hernandez, T.L., Kirwan, J.P., Catalano, P.M., Friedman, J.E., 2007. Cellular Mechanisms for Insulin Resistance in Normal Pregnancy and Gestational Diabetes. Diabetes Care 30, S112–S119. https://doi.org/10.2337/dc07-s202

Barbour, L.A., Shao, J., Qiao, L., Pulawa, L.K., Jensen, D.R., Bartke, A., Garrity, M., Draznin, B., Friedman, J.E., 2002. Human placental growth hormone causes severe insulin resistance in transgenic mice. Am. J. Obstet. Gynecol. 186, 512–517. https://doi.org/10.1067/mob.2002.121256

Baron, A.D., Schaeffer, L., Shragg, P., Kolterman, O.G., 1987. Role of hyperglucagonemia in maintenance of increased rates of hepatic glucose output in type II diabetics. Diabetes 36, 274–283. https://doi.org/10.2337/diab.36.3.274

Basukala, P., Jha, B., Yadav, B.K., Shrestha, P.K., 2018. Determination of Insulin Resistance and Beta-Cell Function Using Homeostatic Model Assessment in Type 2 Diabetic Patients at Diagnosis. J Diabetes Metab 09. https://doi.org/10.4172/2155-6156.1000790

Behan, D.P., De Souza, E.B., Lowry, P.J., Potter, E., Sawchenko, P., Vale, W.W., 1995. Corticotropin releasing factor (CRF) binding protein: a novel regulator of CRF and related peptides. Front Neuroendocrinol 16, 362–382. https://doi.org/10.1006/frne.1995.1013

Behboudi-Gandevani, S., Amiri, M., Bidhendi Yarandi, R., Ramezani Tehrani, F., 2019. The impact of diagnostic criteria for gestational diabetes on its prevalence: a systematic review and meta-analysis. Diabetol Metab Syndr 11. https://doi.org/10.1186/s13098-019-0406-1

Bellamy, L., Casas, J.-P., Hingorani, A.D., Williams, D., 2009a. Type 2 diabetes mellitus after gestational diabetes: a systematic review and meta-analysis. The Lancet 373, 1773–1779. https://doi.org/10.1016/S0140-6736(09)60731-5

Beloff-Chain, A., Hawthorn, J., 1976. The release of insulin from pancreatic islets of lean and obese mice stimulated in vitro by pituitary glands from obese mice and by high glucose concentrations. FEBS Letters 64, 214–217. https://doi.org/10.1016/0014-5793(76)80286-4

Benner, C., van der Meulen, T., Cacéres, E., Tigyi, K., Donaldson, C.J., Huising, M.O., 2014. The transcriptional landscape of mouse beta cells compared to human beta cells reveals notable species differences in long non-coding RNA and protein-coding gene expression. BMC Genomics 15. https://doi.org/10.1186/1471-2164-15-620

Bensley, R.R., 1911. Studies on the pancreas of the guinea pig. American Journal of Anatomy 12, 297–388. https://doi.org/10.1002/aja.1000120304

Berberoglu, Z., 2019. Pathophysiology of Gestational Diabetes Mellitus [WWW Document]. European Medical Journal. URL https://www.emjreviews.com/diabetes/article/pathophysiology-of-gestational-diabetes-mellitus/ (accessed 12.16.19).

Bernal, A.L., 2001. Overview of Current Research in Parturition. Experimental Physiology 86, 213–222. https://doi.org/10.1113/eph8602178

Blum, J.L., Chen, L.-C., Zelikoff, J.T., 2017. Exposure to Ambient Particulate Matter during Specific Gestational Periods Produces Adverse Obstetric Consequences in Mice. Environ Health Perspect 125. https://doi.org/10.1289/EHP1029

Bock, T., Pakkenberg, B., Buschard, K., 2003. Increased Islet Volume but Unchanged Islet Number in ob/ob Mice. Diabetes 52, 1716–1722. https://doi.org/10.2337/diabetes.52.7.1716

Bonaz, B.L., Bernstein, C.N., 2013. Brain-Gut Interactions in Inflammatory Bowel Disease. Gastroenterology 144, 36–49. https://doi.org/10.1053/j.gastro.2012.10.003

Bonfiglio, J.J., Inda, C., Refojo, D., Holsboer, F., Arzt, E., Silberstein, S., 2011. The Corticotropin-Releasing Hormone Network and the Hypothalamic-Pituitary-Adrenal Axis: Molecular and Cellular Mechanisms Involved. NEN 94, 12–20. https://doi.org/10.1159/000328226

Bonomo, M., Corica, D., Mion, E., Gonçalves, D., Motta, G., Merati, R., Ragusa, A., Morabito, A., 2005. Evaluating the therapeutic approach in pregnancies complicated by borderline glucose intolerance: a randomized clinical trial. Diabet. Med. 22, 1536–1541. https://doi.org/10.1111/j.1464-5491.2005.01690.x

Boorse, G.C., Denver, R.J., 2006. Widespread tissue distribution and diverse functions of corticotropin-releasing factor and related peptides. General and Comparative Endocrinology 146, 9–18. https://doi.org/10.1016/j.ygcen.2005.11.014

Borg, M.L., Massart, J., Schönke, M., De Castro Barbosa, T., Guo, L., Wade, M., Alsina-Fernandez, J., Miles, R., Ryan, A., Bauer, S., Coskun, T., O'Farrell, E., Niemeier, E.M., Chibalin, A.V., Krook, A., Karlsson, H.K., Brozinick, J.T., Zierath, J.R., 2019. Modified UCN2 Peptide Acts as an Insulin Sensitizer in Skeletal Muscle of Obese Mice. Diabetes 68, 1403–1414. https://doi.org/10.2337/db18-1237

Bornstein, S.R., Webster, E.L., Torpy, D.J., Richman, S.J., Mitsiades, N., Igel, M., Lewis, D.B., Rice, K.C., Joost, H.G., Tsokos, M., Chrousos, G.P., 1998. Chronic Effects of a Nonpeptide Corticotropin-Releasing Hormone Type I Receptor Antagonist on Pituitary-Adrenal Function, Body Weight, and Metabolic Regulation. Endocrinology 139, 1546–1555. https://doi.org/10.1210/endo.139.4.5938

Borst, S.E., 2004. The role of TNF-alpha in insulin resistance. Endocrine 23, 177–182. https://doi.org/10.1385/ENDO:23:2-3:177

Bosco, D., Armanet, M., Morel, P., Niclauss, N., Sgroi, A., Muller, Y.D., Giovannoni, L., Parnaud, G., Berney, T., 2010. Unique Arrangement of α- and β-Cells in Human Islets of Langerhans. Diabetes 59, 1202–1210. https://doi.org/10.2337/db09-1177

Bowe, J.E., Foot, V.L., Amiel, S.A., Huang, G.C., Lamb, M.W., Lakey, J., Jones, P.M., Persaud, S.J., 2012. GPR54 peptide agonists stimulate insulin secretion from murine, porcine and human islets. Islets 4, 20–23. https://doi.org/10.4161/isl.18261

Bowe, J.E., Franklin, Z.J., Hauge-Evans, A.C., King, A.J., Persaud, S.J., Jones, P.M., 2014. METABOLIC PHENOTYPING GUIDELINES: Assessing glucose homeostasis in rodent models. Journal of Endocrinology 222, G13–G25. https://doi.org/10.1530/JOE-14-0182

Bowe, J.E., Hill, T.G., Hunt, K.F., Smith, L.I.F., Simpson, S.J.S., Amiel, S.A., Jones, P.M., 2019. A role for placental kisspeptin in **β** cell adaptation to pregnancy. JCI Insight 4. https://doi.org/10.1172/jci.insight.124540

Bowe, J.E., King, A.J., Kinsey-Jones, J.S., Foot, V.L., Li, X.F., O'Byrne, K.T., Persaud, S.J., Jones, P.M., 2009. Kisspeptin stimulation of insulin secretion: mechanisms of action in mouse islets and rats. Diabetologia 52, 855. https://doi.org/10.1007/s00125-009-1283-1

Brelje, T.C., Scharp, D.W., Lacy, P.E., Ogren, L., Talamantes, F., Robertson, M., Friesen, H.G., Sorenson, R.L., 1993. Effect of homologous placental lactogens, prolactins, and growth hormones on islet B-cell division and insulin secretion in rat, mouse, and human islets: implication for placental lactogen regulation of islet function during pregnancy. Endocrinology 132, 879–887. https://doi.org/10.1210/endo.132.2.8425500

Brink, H.S., van der Lely, A.J., van der Linden, J., 2016. The potential role of biomarkers in predicting gestational diabetes. Endocr Connect 5, R26–R34. https://doi.org/10.1530/EC-16-0033

Buchanan, T.A., 2001. Pancreatic B-Cell Defects in Gestational Diabetes: Implications for the Pathogenesis and Prevention of Type 2 Diabetes. J Clin Endocrinol Metab 86, 989–993. https://doi.org/10.1210/jcem.86.3.7339

Buchanan, T.A., Xiang, A.H., Page, K.A., 2012. Gestational Diabetes Mellitus: Risks and Management during and after Pregnancy. Nat Rev Endocrinol 8, 639–649. https://doi.org/10.1038/nrendo.2012.96

Buckinx, R., Adriaensen, D., Van Nassauw, L., Timmermans, J.-P., 2011. Corticotrophin-Releasing Factor, Related Peptides, and Receptors in the Normal and Inflamed Gastrointestinal Tract. Front. Neurosci. 5. https://doi.org/10.3389/fnins.2011.00054

Butler, A.E., Cao-Minh, L., Galasso, R., Rizza, R.A., Corradin, A., Cobelli, C., Butler, P.C., 2010. Adaptive changes in pancreatic beta cell fractional area and beta cell turnover in human pregnancy. Diabetologia 53, 2167–2176. https://doi.org/10.1007/s00125-010-1809-6

Cabrera, O., Berman, D.M., Kenyon, N.S., Ricordi, C., Berggren, P.-O., Caicedo, A., 2006. The unique cytoarchitecture of human pancreatic islets has implications for islet cell function. Proceedings of the National Academy of Sciences 103, 2334–2339. https://doi.org/10.1073/pnas.0510790103

Cairano, E.S.D., Moretti, S., Marciani, P., Sacchi, V.F., Castagna, M., Davalli, A., Folli, F., Perego, C., 2016. Neurotransmitters and Neuropeptides: New Players in the Control of Islet of Langerhans' Cell Mass and Function. Journal of Cellular Physiology 231, 756–767. https://doi.org/10.1002/jcp.25176

Campbell, E.A., Linton, E.A., Wolfe, C.D.A., Scraggs, P.R., Jones, M.T., Lowry, P.J., 1987. Plasma Corticotropin-Releasing Hormone Concentrations During Pregnancy and Parturition. None 64, 1054–1059. https://doi.org/10.1210/jcem-64-5-1054

Cantley, J., 2014. The control of insulin secretion by adipokines: current evidence for adipocyte-beta cell endocrine signalling in metabolic homeostasis. Mamm Genome 25, 442–454. https://doi.org/10.1007/s00335-014-9538-7

Carter, J.D., Dula, S.B., Corbin, K.L., Wu, R., Nunemaker, C.S., 2009. A Practical Guide to Rodent Islet Isolation and Assessment. Biol Proced Online 11, 3–31. https://doi.org/10.1007/s12575-009-9021-0

Catalano, P.M., 2014. Trying to understand gestational diabetes. Diabetic Medicine 31, 273–281. https://doi.org/10.1111/dme.12381

Catalano, P.M., Hoegh, M., Minium, J., Huston-Presley, L., Bernard, S., Kalhan, S., Hauguel-De Mouzon, S., 2006. Adiponectin in human pregnancy: implications for regulation of glucose and lipid metabolism. Diabetologia 49, 1677–1685. https://doi.org/10.1007/s00125-006-0264-x

Catalano, P.M., Nizielski, S.E., Shao, J., Preston, L., Qiao, L., Friedman, J.E., 2002. Downregulated IRS-1 and PPARgamma in obese women with gestational diabetes: relationship to FFA during pregnancy. Am. J. Physiol. Endocrinol. Metab. 282, E522-533. https://doi.org/10.1152/ajpendo.00124.2001

Catalano, P.M., Tyzbir, E.D., Wolfe, R.R., Calles, J., Roman, N.M., Amini, S.B., Sims, E.A., 1993. Carbohydrate metabolism during pregnancy in control subjects and women with gestational diabetes. Am. J. Physiol. 264, E60-67. https://doi.org/10.1152/ajpendo.1993.264.1.E60

Ćetković, A., Miljic, D., Ljubić, A., Patterson, M., Ghatei, M., Stamenković, J., Nikolic-Djurovic, M., Pekic, S., Doknic, M., Glišić, A., Bloom, S., Popovic, V., 2012. Plasma Kisspeptin Levels in Pregnancies with Diabetes and Hypertensive Disease as a Potential Marker of Placental Dysfunction and Adverse Perinatal Outcome. Endocrine Research 37, 78–88. https://doi.org/10.3109/07435800.2011.639319

Charles, C.J., Jardine, D.L., Rademaker, M.T., Richards, A.M., 2010. Urocortin 2 induces potent long-lasting inhibition of cardiac sympathetic drive despite baroreflex activation in conscious sheep. Journal of Endocrinology 204, 181–189. https://doi.org/10.1677/JOE-08-0505

Chatoo, M., Li, Y., Ma, Z., Coote, J., Du, J., Chen, X., 2018. Involvement of Corticotropin-Releasing Factor and Receptors in Immune Cells in Irritable Bowel Syndrome. Front. Endocrinol. 9. https://doi.org/10.3389/fendo.2018.00021

Chatzaki, E., Margioris, A.N., Gravanis, A., 2002. Expression and regulation of corticotropin-releasing hormone binding protein (CRH-BP) in rat adrenals. Journal of Neurochemistry 80, 81–90. https://doi.org/10.1046/j.0022-3042.2001.00667.x

Chen, A., Blount, A., Vaughan, J., Brar, B., Vale, W., 2004. Urocortin II Gene Is Highly Expressed in Mouse Skin and Skeletal Muscle Tissues: Localization, Basal Expression in Corticotropin-Releasing Factor Receptor (CRFR) 1- and CRFR2-Null Mice, and Regulation by Glucocorticoids. Endocrinology 145, 2445–2457. https://doi.org/10.1210/en.2003-1570

Chen, A., Brar, B., Choi, C.S., Rousso, D., Vaughan, J., Kuperman, Y., Kim, S.N., Donaldson, C., Smith, S.M., Jamieson, P., Li, C., Nagy, T.R., Shulman, G.I., Lee, K.-F., Vale, W., 2006. Urocortin 2 modulates glucose utilization and insulin sensitivity in skeletal muscle. Proc. Natl. Acad. Sci. U.S.A. 103, 16580–16585. https://doi.org/10.1073/pnas.0607337103

Chen, A.M., Perrin, M.H., DiGruccio, M.R., Vaughan, J.M., Brar, B.K., Arias, C.M., Lewis, K.A., Rivier, J.E., Sawchenko, P.E., Vale, W.W., 2005. A soluble mouse brain splice variant of type 2α corticotropin-releasing factor (CRF) receptor binds ligands and modulates their activity. PNAS 102, 2620–2625. https://doi.org/10.1073/pnas.0409583102

Chen, H., Kleinberger, J.W., Takane, K.K., Salim, F., Fiaschi-Taesch, N., Pappas, K., Parsons, R., Jiang, J., Zhang, Y., Liu, H., Wang, P., Bender, A.S., Frank, S.J., Stewart, A.F., 2015. Augmented Stat5 Signaling Bypasses Multiple Impediments to Lactogen-Mediated Proliferation in Human β-Cells. Diabetes 64, 3784–3797. https://doi.org/10.2337/db15-0083

Chen, J., Tan, B., Karteris, E., Zervou, S., Digby, J., Hillhouse, E.W., Vatish, M., Randeva, H.S., 2006. Secretion of adiponectin by human placenta: differential modulation of adiponectin and its receptors by cytokines. Diabetologia 49, 1292–1302. https://doi.org/10.1007/s00125-006-0194-7

Chen, L., Magliano, D.J., Zimmet, P.Z., 2012. The worldwide epidemiology of type 2 diabetes mellitus—present and future perspectives. Nature Reviews Endocrinology 8, 228–236. https://doi.org/10.1038/nrendo.2011.183

Chen, R., Lewis, K.A., Perrin, M.H., Vale, W.W., 1993. Expression cloning of a human corticotropin-releasing-factor receptor. Proc. Natl. Acad. Sci. U.S.A. 90, 8967–8971.

Chen, Y.L., Mansbach, R.S., Winter, S.M., Brooks, E., Collins, J., Corman, M.L., Dunaiskis, A.R., Faraci, W.S., Gallaschun, R.J., Schmidt, A., Schulz, D.W., 1997. Synthesis and Oral Efficacy of a 4-(Butylethylamino)pyrrolo[2,3- *d*]pyrimidine: A Centrally Active Corticotropin-Releasing Factor $_1$ Receptor Antagonist. J. Med. Chem. 40, 1749–1754. https://doi.org/10.1021/jm960861b

Chia, R., Achilli, F., Festing, M.F.W., Fisher, E.M.C., 2005. The origins and uses of mouse outbred stocks. Nature Genetics 37, 1181–1186. https://doi.org/10.1038/ng1665

Cho, N.H., Shaw, J.E., Karuranga, S., Huang, Y., da Rocha Fernandes, J.D., Ohlrogge, A.W., Malanda, B., 2018. IDF Diabetes Atlas: Global estimates of diabetes prevalence for 2017 and projections for 2045. Diabetes Research and Clinical Practice 138, 271–281. https://doi.org/10.1016/j.diabres.2018.02.023

Clarke, H., Dhillo, W.S., Jayasena, C.N., 2015. Comprehensive Review on Kisspeptin and Its Role in Reproductive Disorders. Endocrinol Metab (Seoul) 30, 124–141. https://doi.org/10.3803/EnM.2015.30.2.124

Clausen, T.D., Mathiesen, E.R., Hansen, T., Pedersen, O., Jensen, D.M., Lauenborg, J., Damm, P., 2008. High Prevalence of Type 2 Diabetes and Pre-Diabetes in Adult Offspring of Women With Gestational Diabetes Mellitus or Type 1 Diabetes: The role of intrauterine hyperglycemia. Diabetes Care 31, 340–346. https://doi.org/10.2337/dc07-1596

Clutter, W.E., Rizza, R.A., Gerich, J.E., Cryer, P.E., 1988. Regulation of glucose metabolism by sympathochromaffin catecholamines. Diabetes/metabolism research and reviews 4, 1–15. https://doi.org/10.1002/dmr.5610040104

Colberg, S.R., Sigal, R.J., Yardley, J.E., Riddell, M.C., Dunstan, D.W., Dempsey, P.C., Horton, E.S., Castorino, K., Tate, D.F., 2016. Physical Activity/Exercise and Diabetes: A Position Statement of the American Diabetes Association. Diabetes Care 39, 2065–2079. https://doi.org/10.2337/dc16-1728

Consensus Panel, I.A. of D. and P.S.G.C., 2010. International Association of Diabetes and Pregnancy Study Groups Recommendations on the Diagnosis and Classification of Hyperglycemia in Pregnancy. Diabetes Care 33, 676–682. https://doi.org/10.2337/dc09-1848

Cook, D.L., Hales, N., 1984. Intracellular ATP directly blocks K+ channels in pancreatic B-cells. Nature 311, 271–273. https://doi.org/10.1038/311271a0

Coste, S.C., Quintos, R.F., Stenzel-Poore, M.P., 2002. Corticotropin-Releasing Hormone-Related Peptides and Receptors: Emergent Regulators of Cardiovascular Adaptations to Stress.

Trends in Cardiovascular Medicine 12, 176–182. https://doi.org/10.1016/S1050-1738(02)00157-3
Coustan, D.R., 2007. Pharmacological Management of Gestational Diabetes: An overview. Diabetes Care 30, S206–S208. https://doi.org/10.2337/dc07-s217
Curry, D.L., Bennett, L.L., Grodsky, G.M., 1968. Dynamics of Insulin Secretion by the Perfused Rat Pancreas. Endocrinology 83, 572–584. https://doi.org/10.1210/endo-83-3-572
Da Silva Xavier, G., 2018. The Cells of the Islets of Langerhans. J Clin Med 7. https://doi.org/10.3390/jcm7030054
Dautzenberg, F.M., Hauger, R.L., 2002. The CRF peptide family and their receptors: yet more partners discovered. Trends in Pharmacological Sciences 23, 71–77. https://doi.org/10.1016/S0165-6147(02)01946-6
Davidson, S.M., Rybka, A.E., Townsend, P.A., 2009. The powerful cardioprotective effects of urocortin and the corticotropin releasing hormone (CRH) family. Biochemical Pharmacology 77, 141–150. https://doi.org/10.1016/j.bcp.2008.08.033
Davis, M.E., Pemberton, C.J., Yandle, T.G., Fisher, S.F., Lainchbury, J.G., Frampton, C.M., Rademaker, M.T., Richards, A.M., 2007. Urocortin 2 Infusion in Healthy Humans: Hemodynamic, Neurohormonal, and Renal Responses. Journal of the American College of Cardiology 49, 461–471. https://doi.org/10.1016/j.jacc.2006.09.035
de Roux, N., Genin, E., Carel, J.-C., Matsuda, F., Chaussain, J.-L., Milgrom, E., 2003. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. Proc Natl Acad Sci U S A 100, 10972–10976. https://doi.org/10.1073/pnas.1834399100
De Vos, A., Heimberg, H., Quartier, E., Huypens, P., Bouwens, L., Pipeleers, D., Schuit, F., 1995. Human and rat beta cells differ in glucose transporter but not in glucokinase gene expression. J Clin Invest 96, 2489–2495.
Dedic, N., Chen, A., Deussing, J.M., 2018. The CRF Family of Neuropeptides and their Receptors - Mediators of the Central Stress Response. Curr Mol Pharmacol 11, 4–31. https://doi.org/10.2174/1874467210666170302104053
Demirci, C., Ernst, S., Alvarez-Perez, J.C., Rosa, T., Valle, S., Shridhar, V., Casinelli, G.P., Alonso, L.C., Vasavada, R.C., García-Ocana, A., 2012. Loss of HGF/c-Met Signaling in Pancreatic β-Cells Leads to Incomplete Maternal β-Cell Adaptation and Gestational Diabetes Mellitus. Diabetes 61, 1143–1152. https://doi.org/10.2337/db11-1154
Dermitzaki, E., Tsatsanis, C., Minas, V., Chatzaki, E., Charalampopoulos, I., Venihaki, M., Androulidaki, A., Lambropoulou, M., Spiess, J., Michalodimitrakis, E., Gravanis, A., Margioris, A.N., 2007. Corticotropin-Releasing Factor (CRF) and the Urocortins Differentially Regulate Catecholamine Secretion in Human and Rat Adrenals, in a CRF Receptor Type-Specific Manner. Endocrinology 148, 1524–1538. https://doi.org/10.1210/en.2006-0967
Dhillo, W.S., Savage, P., Murphy, K.G., Chaudhri, O.B., Patterson, M., Nijher, G.M., Foggo, V.M., Dancey, G.S., Mitchell, H., Seckl, M.J., Ghatei, M.A., Bloom, S.R., 2006. Plasma kisspeptin is raised in patients with gestational trophoblastic neoplasia and falls during treatment. Am. J. Physiol. Endocrinol. Metab. 291, E878-884. https://doi.org/10.1152/ajpendo.00555.2005
Diabetes UK, 2019. Diabetes Facts and Stats [WWW Document]. Diabetes Facts and Stats. URL https://www.diabetes.org.uk/resources-s3/2019-02/1362B_Facts%20and%20stats%20Update%20Jan%202019_LOW%20RES_EXTERNAL.pdf
Dickens, L.T., Thomas, C.C., 2019. Updates in Gestational Diabetes Prevalence, Treatment, and Health Policy. Curr Diab Rep 19, 33. https://doi.org/10.1007/s11892-019-1147-0
Dishi, M., Hevner, K., Qiu, C., Fida, N.G., Abetew, D.F., Williams, M.A., Enquobahrie, D.A., 2015. Early Pregnancy Maternal Hepatocyte Growth Factor and Risk of Gestational Diabetes. Br J Med Med Res 9. https://doi.org/10.9734/BJMMR/2015/18632
Dolenšek, J., Rupnik, M.S., Stožer, A., 2015. Structural similarities and differences between the human and the mouse pancreas. Islets 7, e1024405. https://doi.org/10.1080/19382014.2015.1024405
Dornhorst, A., Paterson, C.M., Nicholls, J.S.D., Wadsworth, J., Chiu, D.C., Elkeles, R.S., Johnston, D.G., Beard, R.W., 1992. High Prevalence of Gestational Diabetes in Women

from Ethnic Minority Groups. Diabetic Medicine 9, 820–825. https://doi.org/10.1111/j.1464-5491.1992.tb01900.x
Doucette, T.A., Ryan, C.L., Tasker, R.A., 2000. Use of osmotic minipumps for sustained drug delivery in rat pups: effects on physical and neurobehavioural development. Physiol. Behav. 71, 207–212. https://doi.org/10.1016/s0031-9384(00)00315-2
Drynda, R., Persaud, S.J., Bowe, J.E., Jones, P.M., 2018. The Placental Secretome: Identifying Potential Cross-Talk Between Placenta and Islet β-Cells. Cell. Physiol. Biochem. 45, 1165–1171. https://doi.org/10.1159/000487357
Drynda, R., Peters, C.J., Jones, P.M., Bowe, J.E., 2015. The role of non-placental signals in the adaptation of islets to pregnancy. Horm. Metab. Res. 47, 64–71. https://doi.org/10.1055/s-0034-1395691
Dunning, B.E., Gerich, J.E., 2007. The Role of α-Cell Dysregulation in Fasting and Postprandial Hyperglycemia in Type 2 Diabetes and Therapeutic Implications. Endocr Rev 28, 253–283. https://doi.org/10.1210/er.2006-0026
Eisenbarth, G.S., 1986. Type I Diabetes Mellitus. New England Journal of Medicine 314, 1360–1368. https://doi.org/10.1056/NEJM198605223142106
Ekinci, E.I., Torkamani, N., Ramchand, S.K., Churilov, L., Sikaris, K.A., Lu, Z.X., Houlihan, C.A., 2017. Higher maternal serum prolactin levels are associated with reduced glucose tolerance during pregnancy. J Diabetes Investig 8, 697–700. https://doi.org/10.1111/jdi.12634
Eliasson, L., Renström, E., Ämmälä, C., Berggren, P.-O., Bertorello, A.M., Bokvist, K., Chibalin, A., Deeney, J.T., Flatt, P.R., Gäbel, J., Gromada, J., Larsson, O., Lindström, P., Rhodes, C.J., Rorsman, P., 1996. PKC-Dependent Stimulation of Exocytosis by Sulfonylureas in Pancreaticβ Cells. Science 271, 813–815. https://doi.org/10.1126/science.271.5250.813
Erion, K., Corkey, B.E., 2018. β-Cell Failure or β-Cell Abuse? Front. Endocrinol. 9. https://doi.org/10.3389/fendo.2018.00532
Ernst, S., Demirci, C., Valle, S., Velazquez-Garcia, S., Garcia-Ocaña, A., 2011. Mechanisms in the adaptation of maternal β-cells during pregnancy. Diabetes Manag (Lond) 1, 239–248. https://doi.org/10.2217/dmt.10.24
Fekete, É.M., Zorrilla, E.P., 2007. Physiology, pharmacology, and therapeutic relevance of urocortins in mammals: Ancient CRF paralogs. Frontiers in Neuroendocrinology 28, 1–27. https://doi.org/10.1016/j.yfrne.2006.09.002
Feng, Y., Feng, Q., Qu, H., Song, X., Hu, J., Xu, X., Zhang, L., Yin, S., 2020. Stress adaptation is associated with insulin resistance in women with gestational diabetes mellitus. Nutrition & Diabetes 10, 1–4. https://doi.org/10.1038/s41387-020-0107-8
Ferrara, A., 2007. Increasing Prevalence of Gestational Diabetes Mellitus: A public health perspective. Diabetes Care 30, S141–S146. https://doi.org/10.2337/dc07-s206
Festing, M.F.W., 1979. Inbred Strains in Biomedical Research. Macmillan Education UK, London. https://doi.org/10.1007/978-1-349-03816-9
Fischer, S., Hanefeld, M., Haffner, S.M., Fusch, C., Schwanebeck, U., Köhler, C., Fücker, K., Julius, U., 2002. Insulin-resistant patients with type 2 diabetes mellitus have higher serum leptin levels independently of body fat mass. Acta Diabetologica 39, 105–110. https://doi.org/10.1007/s005920200027
Freemark, M., 2006. Regulation of Maternal Metabolism by Pituitary and Placental Hormones: Roles in Fetal Development and Metabolic Programming. HRP 65, 41–49. https://doi.org/10.1159/000091505
Freemark, M., Avril, I., Fleenor, D., Driscoll, P., Petro, A., Opara, E., Kendall, W., Oden, J., Bridges, S., Binart, N., Breant, B., Kelly, P.A., 2002. Targeted Deletion of the PRL Receptor: Effects on Islet Development, Insulin Production, and Glucose Tolerance. Endocrinology 143, 1378–1385. https://doi.org/10.1210/endo.143.4.8722
Friedman, J.E., Ishizuka, T., Shao, J., Huston, L., Highman, T., Catalano, P., 1999. Impaired glucose transport and insulin receptor tyrosine phosphorylation in skeletal muscle from obese women with gestational diabetes. Diabetes 48, 1807–1814. https://doi.org/10.2337/diabetes.48.9.1807
Frim, D.M., Emanuel, R.L., Robinson, B.G., Smas, C.M., Adler, G.K., Majzoub, J.A., 1988. Characterization and gestational regulation of corticotropin-releasing hormone messenger RNA in human placenta. J Clin Invest 82, 287–292.

Gangestad, S.W., Caldwell Hooper, A.E., Eaton, M.A., 2012. On the function of placental corticotropin-releasing hormone: a role in maternal-fetal conflicts over blood glucose concentrations. Biological Reviews 87, 856–873. https://doi.org/10.1111/j.1469-185X.2012.00226.x

Gao, M.H., Giamouridis, D., Lai, N.C., Guo, T., Xia, B., Kim, Y.C., Huu, V.A.N., Skowronska-Krawczyk, D., Lantier, L., Bhargava, R., Hammond, H.K., 2019. Urocortin 2 Gene Transfer Improves Glycemic Control and Reduces Retinopathy and Mortality in Murine Insulin Deficiency. Mol Ther Methods Clin Dev 17, 220–233. https://doi.org/10.1016/j.omtm.2019.12.002

Gao, M.H., Giamouridis, D., Lai, N.C., Walenta, E., Paschoal, V.A., Kim, Y.C., Miyanohara, A., Guo, T., Liao, M., Liu, L., Tan, Z., Ciaraldi, T.P., Schenk, S., Bhargava, A., Oh, D.Y., Hammond, H.K., 2016. One-time injection of AAV8 encoding urocortin 2 provides long-term resolution of insulin resistance. JCI Insight 1. https://doi.org/10.1172/jci.insight.88322

Garcia-Ocaña, A., Takane, K.K., Syed, M.A., Philbrick, W.M., Vasavada, R.C., Stewart, A.F., 2000. Hepatocyte growth factor overexpression in the islet of transgenic mice increases beta cell proliferation, enhances islet mass, and induces mild hypoglycemia. J. Biol. Chem. 275, 1226–1232. https://doi.org/10.1074/jbc.275.2.1226

Genevay, M., Pontes, H., Meda, P., 2010. Beta cell adaptation in pregnancy: a major difference between humans and rodents? Diabetologia 53, 2089–2092. https://doi.org/10.1007/s00125-010-1848-z

Getahun, D., Fassett, M.J., Jacobsen, S.J., 2010. Gestational diabetes: risk of recurrence in subsequent pregnancies. American Journal of Obstetrics & Gynecology 203, 467.e1-467.e6. https://doi.org/10.1016/j.ajog.2010.05.032

Gey, G.O., Gey, M.K., 1936. The Maintenance of Human Normal Cells and Tumor Cells in Continuous Culture: I. Preliminary Report: Cultivation of Mesoblastic Tumors and Normal Tissue and Notes on Methods of Cultivation. The American Journal of Cancer 27, 45–76. https://doi.org/10.1158/ajc.1936.45

Gillis, K.D., Misler, S., 1992. Single cell assay of exocytosis from pancreatic islet B cells. Pflugers Arch. 420, 121–123. https://doi.org/10.1007/BF00378654

Gilman, A.G., 1987. G proteins: transducers of receptor-generated signals. Annu. Rev. Biochem. 56, 615–649. https://doi.org/10.1146/annurev.bi.56.070187.003151

Gilmartin, A. "Bird" H., Ural, S.H., Repke, J.T., 2008. Gestational Diabetes Mellitus. Rev Obstet Gynecol 1, 129–134.

Goldsmith, S.J., 1975. Radioimmunoassay: Review of basic principles. Seminars in Nuclear Medicine 5, 125–152. https://doi.org/10.1016/S0001-2998(75)80028-6

Gorvin, C.M., 2015. The prolactin receptor: Diverse and emerging roles in pathophysiology. J Clin Transl Endocrinol 2, 85–91. https://doi.org/10.1016/j.jcte.2015.05.001

Gotoh, M., Maki, T., Kiyoizumi, T., Satomi, S., Monaco, A.P., 1985. AN IMPROVED METHOD FOR ISOLATION OF MOUSE PANCREATIC ISLETS. Transplantation 40, 437.

Gould, G.W., Holman, G.D., 1993. The glucose transporter family: structure, function and tissue-specific expression. Biochem J 295, 329–341.

Goyvaerts, L., Schraenen, A., Schuit, F., 2016. Serotonin competence of mouse beta cells during pregnancy. Diabetologia 59, 1356–1363. https://doi.org/10.1007/s00125-016-3951-2

Gracie, T.J., 2015. Conditional knockout of Crhr2 in serotonergic neurons decreases the expression of htr1a in the dorsal raphe nucleus.

Grammatopoulos, D.K., 2012. Insights into mechanisms of corticotropin-releasing hormone receptor signal transduction: CRH-R signalling. British Journal of Pharmacology 166, 85–97. https://doi.org/10.1111/j.1476-5381.2011.01631.x

Grammatopoulos, D.K., 2008. Placental Corticotrophin-Releasing Hormone and its Receptors in Human Pregnancy and Labour: Still a Scientific Enigma. Journal of Neuroendocrinology 20, 432–438. https://doi.org/10.1111/j.1365-2826.2008.01660.x

Grammatopoulos, D.K., Chrousos, G.P., 2002. Functional characteristics of CRH receptors and potential clinical applications of CRH-receptor antagonists. Trends in Endocrinology & Metabolism 13, 436–444. https://doi.org/10.1016/S1043-2760(02)00670-7

Grammatopoulos, D.K., Randeva, H.S., Levine, M.A., Kanellopoulou, K.A., Hillhouse, E.W., 2001. Rat cerebral cortex corticotropin-releasing hormone receptors: evidence for

receptor coupling to multiple G-proteins. J. Neurochem. 76, 509–519. https://doi.org/10.1046/j.1471-4159.2001.00067.x
Gravanis, A., Margioris, A.N., 2005. The corticotropin-releasing factor (CRF) family of neuropeptides in inflammation: potential therapeutic applications. Curr. Med. Chem. 12, 1503–1512. https://doi.org/10.2174/0929867054039008
Green, I.C., Taylor, K.W., 1972. Effects of pregnancy in the rat on the size and insulin secretory response of the islets of langerhans. Journal of Endocrinology 54, 317–325. https://doi.org/10.1677/joe.0.0540317
Griffin, M.E., Coffey, M., Johnson, H., Scanlon, P., Foley, M., Stronge, J., O'Meara, N.M., Firth, R.G., 2000. Universal vs. risk factor-based screening for gestational diabetes mellitus: detection rates, gestation at diagnosis and outcome. Diabetic Medicine 17, 26–32. https://doi.org/10.1046/j.1464-5491.2000.00214.x
Grigorakis, S.I., Alevizaki, M., Beis, C., Anastasiou, E., Alevizaki, C.C., Souvatzoglou, A., 2000. Hormonal Parameters in Gestational Diabetes mellitus during the Third Trimester: High Glucagon Levels. GOI 49, 106–109. https://doi.org/10.1159/000010225
Grino, M., Chrousos, G.P., Margioris, A.N., 1987a. The corticotropin releasing hormone gene is expressed in human placenta. Biochem. Biophys. Res. Commun. 148, 1208–1214. https://doi.org/10.1016/s0006-291x(87)80261-9
Gross, K.J., Pothoulakis, C., 2007. Role of neuropeptides in inflammatory bowel disease. Inflamm Bowel Dis 13, 918–932. https://doi.org/10.1002/ibd.20129
Guillam, M.-T., Hümmler, E., Schaerer, E., Wu, J.-Y., Birnbaum, M.J., Beermann, F., Schmidt, A., Dériaz, N., Thorens, B., 1997. Early diabetes and abnormal postnatal pancreatic islet development in mice lacking Glut-2. Nature Genetics 17, 327–330. https://doi.org/10.1038/ng1197-327
Guillemin, R., Rosenberg, B., 1955. Humoral hypothalamic control of anterior pituitary: a study with combined tissue cultures. Endocrinology 57, 599–607. https://doi.org/10.1210/endo-57-5-599
Hales, C.N., Milner, R.D.G., 1968. The role of sodium and potassium in insulin secretion from rabbit pancreas. The Journal of Physiology 194, 725–743. https://doi.org/10.1113/jphysiol.1968.sp008433
HAPO Study Cooperative Research Group, Metzger, B.E., Lowe, L.P., Dyer, A.R., Trimble, E.R., Chaovarindr, U., Coustan, D.R., Hadden, D.R., McCance, D.R., Hod, M., McIntyre, H.D., Oats, J.J.N., Persson, B., Rogers, M.S., Sacks, D.A., 2008. Hyperglycemia and adverse pregnancy outcomes. N. Engl. J. Med. 358, 1991–2002. https://doi.org/10.1056/NEJMoa0707943
Harper, L.M., Mele, L., Landon, M.B., Carpenter, M.W., Ramin, S.M., Reddy, U.M., Casey, B., Wapner, R.J., Varner, M.W., Thorp, J.M., Sciscione, A., Catalano, P., Harper, M., Saade, G., Caritis, S.N., Sorokin, Y., Peaceman, A.M., Tolosa, J.E., 2016. Carpenter-Coustan Compared With National Diabetes Data Group Criteria for Diagnosing Gestational Diabetes. Obstet Gynecol 127, 893–898. https://doi.org/10.1097/AOG.0000000000001383
Hatting, M., Tavares, C.D.J., Sharabi, K., Rines, A.K., Puigserver, P., 2018. Insulin regulation of gluconeogenesis. Ann N Y Acad Sci 1411, 21–35. https://doi.org/10.1111/nyas.13435
Hauge-Evans, A.C., Richardson, C.C., Milne, H.M., Christie, M.R., Persaud, S.J., Jones, P.M., 2006. A role for kisspeptin in islet function. Diabetologia 49, 2131–2135. https://doi.org/10.1007/s00125-006-0343-z
Hauger, R.L., Grigoriadis, D.E., Dallman, M.F., Plotsky, P.M., Vale, W.W., Dautzenberg, F.M., 2003a. International Union of Pharmacology. XXXVI. Current Status of the Nomenclature for Receptors for Corticotropin-Releasing Factor and Their Ligands. Pharmacol Rev 55, 21–26. https://doi.org/10.1124/pr.55.1.3
Hauger, R.L., Risbrough, V., Brauns, O., Dautzenberg, F.M., 2006. Corticotropin Releasing Factor (CRF) Receptor Signaling in the Central Nervous System: New Molecular Targets. CNS Neurol Disord Drug Targets 5, 453–479.
Hebert, M.F., Ma, X., Naraharisetti, S.B., Krudys, K.M., Umans, J.G., Hankins, G.D.V., Caritis, S.N., Miodovnik, M., Mattison, D.R., Unadkat, J.D., Kelly, E.J., Blough, D., Cobelli, C., Ahmed, M.S., Snodgrass, W.R., Carr, D.B., Easterling, T.R., Vicini, P., 2009. Are We Optimizing Gestational Diabetes Treatment With Glyburide? The Pharmacologic Basis

for Better Clinical Practice. Clinical Pharmacology & Therapeutics 85, 607–614. https://doi.org/10.1038/clpt.2009.5
Hedrich, H.J., Bullock, G.R. (Eds.), 2004. The laboratory mouse, The handbook of experimental animals. Elsevier Academic Press, Amsterdam ; Boston.
Hellman, B., Idahl, L.-Å., Lernmark, Å., Sehlin, J., Täljedal, I.-B., 1974. The pancreatic β-cell recognition of insulin secretagogues: Comparisons of glucose with glyceraldehyde isomers and dihydroxyacetone. Archives of Biochemistry and Biophysics 162, 448–457. https://doi.org/10.1016/0003-9861(74)90204-5
Henderson, C.E., Divon, M.Y., 1998. Combining human placental lactogen with routine glucose challenge tests. Primary Care Update for OB/GYNS 5, 189–190. https://doi.org/10.1016/S1068-607X(98)00112-7
Henquin, J.-C., Lambert, A.E., 1975. Extracellular bicarbonate ions and insulin secretion. Biochimica et Biophysica Acta (BBA) - General Subjects 381, 437–442. https://doi.org/10.1016/0304-4165(75)90251-2
Herman, J.P., McKlveen, J.M., Ghosal, S., Kopp, B., Wulsin, A., Makinson, R., Scheimann, J., Myers, B., 2016. Regulation of the hypothalamic-pituitary-adrenocortical stress response. Compr Physiol 6, 603–621. https://doi.org/10.1002/cphy.c150015
Hillhouse, E.W., Grammatopoulos, D.K., 2001. Characterising the Corticotropin-releasing Hormone (CRH) Receptors Mediating CRH and Urocortin Actions During Human Pregnancy and Labour. Stress 4, 235–246. https://doi.org/10.3109/10253890109014748
Hiroi, N., Wong, M.L., Licinio, J., Park, C., Young, M., Gold, P.W., Chrousos, G.P., Bornstein, S.R., 2001. Expression of corticotropin releasing hormone receptors type I and type II mRNA in suicide victims and controls. Mol Psychiatry 6, 540–546. https://doi.org/10.1038/sj.mp.4000908
Hohmeier, H.E., Newgard, C.B., 2004. Cell lines derived from pancreatic islets. Molecular and Cellular Endocrinology, Endocrine Cell Lines 228, 121–128. https://doi.org/10.1016/j.mce.2004.04.017
Hollenstein, K., de Graaf, C., Bortolato, A., Wang, M.-W., Marshall, F.H., Stevens, R.C., 2014. Insights into the structure of class B GPCRs. Trends Pharmacol Sci 35, 12–22. https://doi.org/10.1016/j.tips.2013.11.001
Homko, C., Sivan, E., Chen, X., Reece, E.A., Boden, G., 2001. Insulin Secretion during and after Pregnancy in Patients with Gestational Diabetes Mellitus. J Clin Endocrinol Metab 86, 568–573. https://doi.org/10.1210/jcem.86.2.7137
Horibe, N., Okamoto, T., Itakura, A., Nakanishi, T., Suzuki, T., Kazeto, S., Tomoda, Y., 1995. Levels of hepatocyte growth factor in maternal serum and amniotic fluid. Am. J. Obstet. Gynecol. 173, 937–942. https://doi.org/10.1016/0002-9378(95)90370-4
Horikoshi, Y., Matsumoto, H., Takatsu, Y., Ohtaki, T., Kitada, C., Usuki, S., Fujino, M., 2003a. Dramatic elevation of plasma metastin concentrations in human pregnancy: metastin as a novel placenta-derived hormone in humans. J. Clin. Endocrinol. Metab. 88, 914–919. https://doi.org/10.1210/jc.2002-021235
Hu, F.B., Manson, J.E., Stampfer, M.J., Colditz, G., Liu, S., Solomon, C.G., Willett, W.C., 2001. Diet, lifestyle, and the risk of type 2 diabetes mellitus in women. N. Engl. J. Med. 345, 790–797. https://doi.org/10.1056/NEJMoa010492
Huang, C., Snider, F., Cross, J.C., 2009. Prolactin receptor is required for normal glucose homeostasis and modulation of beta-cell mass during pregnancy. Endocrinology 150, 1618–1626. https://doi.org/10.1210/en.2008-1003
Huang, L., Li, C., 2000. Leptin: a multifunctional hormone. Cell Res. 10, 81–92. https://doi.org/10.1038/sj.cr.7290038
Huising, M.O., 2020. Paracrine regulation of insulin secretion. Diabetologia 63, 2057–2063. https://doi.org/10.1007/s00125-020-05213-5
Huising, M.O., Pilbrow, A.P., Matsumoto, M., van der Meulen, T., Park, H., Vaughan, J.M., Lee, S., Vale, W.W., 2011. Glucocorticoids Differentially Regulate the Expression of CRFR1 and CRFR2α in MIN6 Insulinoma Cells and Rodent Islets. Endocrinology 152, 138–150. https://doi.org/10.1210/en.2010-0791
Huising, M.O., van der Meulen, T., Vaughan, J.M., Matsumoto, M., Donaldson, C.J., Park, H., Billestrup, N., Vale, W.W., 2010. CRFR1 is expressed on pancreatic beta cells, promotes

beta cell proliferation, and potentiates insulin secretion in a glucose-dependent manner. Proc. Natl. Acad. Sci. U.S.A. 107, 912–917. https://doi.org/10.1073/pnas.0913610107
Ilias, I., Mastorakos, G., 2003. The emerging role of peripheral corticotropin-releasing hormone (CRH). J Endocrinol Invest 26, 364–371. https://doi.org/10.1007/BF03345186
Imamura, F., Mukamal, K.J., Meigs, J.B., Luchsinger, J.A., Ix, J.H., Siscovick, D.S., Mozaffarian, D., 2013. Risk Factors for Type 2 Diabetes Mellitus Preceded by β-Cell Dysfunction, Insulin Resistance, or Both in Older Adults. Am J Epidemiol 177, 1418–1429. https://doi.org/10.1093/aje/kws440
Imperatore, A., Florio, P., Torres, P.B., Torricelli, M., Galleri, L., Toti, P., Occhini, R., Picciolini, E., Vale, W., Petraglia, F., 2006. Urocortin 2 and urocortin 3 are expressed by the human placenta, deciduas, and fetal membranes. American Journal of Obstetrics and Gynecology 195, 288–295. https://doi.org/10.1016/j.ajog.2005.12.048
Izzi-Engbeaya, C., Comninos, A.N., Clarke, S.A., Jomard, A., Yang, L., Jones, S., Abbara, A., Narayanaswamy, S., Eng, P.C., Papadopoulou, D., Prague, J.K., Bech, P., Godsland, I.F., Bassett, P., Sands, C., Camuzeaux, S., Gomez-Romero, M., Pearce, J.T.M., Lewis, M.R., Holmes, E., Nicholson, J.K., Tan, T., Ratnasabapathy, R., Hu, M., Carrat, G., Piemonti, L., Bugliani, M., Marchetti, P., Johnson, P.R., Hughes, S.J., Shapiro, A.M.J., Rutter, G.A., Dhillo, W.S., 2018. The effects of kisspeptin on β-cell function, serum metabolites and appetite in humans. Diabetes, Obesity and Metabolism 20, 2800–2810. https://doi.org/10.1111/dom.13460
J, X., Yh, Z., Yp, C., Xl, Y., J, W., H, Z., Cm, L., 2014. Maternal Circulating Concentrations of Tumor Necrosis Factor-Alpha, Leptin, and Adiponectin in Gestational Diabetes Mellitus: A Systematic Review and Meta-Analysis [WWW Document]. TheScientificWorldJournal. https://doi.org/10.1155/2014/926932
Jamieson, P.M., Cleasby, M.E., Kuperman, Y., Morton, N.M., Kelly, P.A.T., Brownstein, D.G., Mustard, K.J., Vaughan, J.M., Carter, R.N., Hahn, C.N., Hardie, D.G., Seckl, J.R., Chen, A., Vale, W.W., 2011. Urocortin 3 transgenic mice exhibit a metabolically favourable phenotype resisting obesity and hyperglycaemia on a high-fat diet. Diabetologia 54, 2392. https://doi.org/10.1007/s00125-011-2205-6
Johns, E.C., Denison, F.C., Norman, J.E., Reynolds, R.M., 2018a. Gestational Diabetes Mellitus: Mechanisms, Treatment, and Complications. Trends Endocrinol. Metab. 29, 743–754. https://doi.org/10.1016/j.tem.2018.09.004
Jones, P.M., SALMONt, D.M.W., Howell, S.L., 1988. Protein phosphorylation in electrically permeabilized islets of Langerhans 254, 7.
Kaku, K., Fiedorek, F.T., Province, M., Permutt, M.A., 1988. Genetic Analysis of Glucose Tolerance in Inbred Mouse Strains: Evidence for Polygenic Control. Diabetes 37, 707–713. https://doi.org/10.2337/diab.37.6.707
Kanno, T., Suga, S., Nakano, K., Kamimura, N., Wakui, M., 1999. Corticotropin-releasing factor modulation of Ca2+ influx in rat pancreatic beta-cells. Diabetes 48, 1741–1746.
Karlsson, S., Ahrén, B., 1988. Effects of corticotropin-releasing hormone on insulin and glucagon secretion in mice. European Journal of Endocrinology 117, 87–92. https://doi.org/10.1530/acta.0.1170087
Karteris, E., Grammatopoulos, D.K., Randeva, H.S., Hillhouse, E.W., 2001. The Role of Corticotropin-Releasing Hormone Receptors in Placenta and Fetal Membranes during Human Pregnancy. Molecular Genetics and Metabolism 72, 287–296. https://doi.org/10.1006/mgme.2001.3159
Kavalakatt, S., Khadir, A., Madhu, D., Hammad, M., Devarajan, S., Abubaker, J., Al-Mulla, F., Tuomilehto, J., Tiss, A., 2019. Urocortin 3 Levels Are Impaired in Overweight Humans With and Without Type 2 Diabetes and Modulated by Exercise. Front. Endocrinol. 10. https://doi.org/10.3389/fendo.2019.00762
Keck, M.E., Ohl, F., Holsboer, F., Müller, M.B., 2005. Listening to mutant mice: a spotlight on the role of CRF/CRF receptor systems in affective disorders. Neuroscience & Biobehavioral Reviews, Animal Models of Depression and Antidepressant Activity 29, 867–889. https://doi.org/10.1016/j.neubiorev.2005.03.003
Kemp, C.F., Woods, R.J., Lowry, P.J., 1998. The corticotrophin-releasing factor-binding protein: an act of several parts. Peptides 19, 1119–1128. https://doi.org/10.1016/S0196-9781(98)00057-6

Kiank, C., Taché, Y., Larauche, M., 2010. Stress-related modulation of inflammation in experimental models of bowel disease and post-infectious irritable bowel syndrome: Role of corticotropin-releasing factor receptors. Brain, Behavior, and Immunity 24, 41–48. https://doi.org/10.1016/j.bbi.2009.08.006

Kim, C., Newton, K.M., Knopp, R.H., 2002. Gestational diabetes and the incidence of type 2 diabetes: a systematic review. Diabetes Care 25, 1862–1868. https://doi.org/10.2337/diacare.25.10.1862

Kim, H., Toyofuku, Y., Lynn, F.C., Chak, E., Uchida, T., Mizukami, H., Fujitani, Y., Kawamori, R., Miyatsuka, T., Kosaka, Y., Yang, K., Honig, G., van der Hart, M., Kishimoto, N., Wang, J., Yagihashi, S., Tecott, L.H., Watada, H., German, M.S., 2010. Serotonin regulates pancreatic beta cell mass during pregnancy. Nat. Med. 16, 804–808. https://doi.org/10.1038/nm.2173

King, A.J., 2012. The use of animal models in diabetes research. British Journal of Pharmacology 166, 877–894. https://doi.org/10.1111/j.1476-5381.2012.01911.x

Kirwan, J.P., Mouzon, S.H.-D., Lepercq, J., Challier, J.-C., Huston-Presley, L., Friedman, J.E., Kalhan, S.C., Catalano, P.M., 2002a. TNF-α Is a Predictor of Insulin Resistance in Human Pregnancy. Diabetes 51, 2207–2213. https://doi.org/10.2337/diabetes.51.7.2207

Komatsu, M., Takei, M., Ishii, H., Sato, Y., 2013. Glucose-stimulated insulin secretion: A newer perspective. J Diabetes Investig 4, 511–516. https://doi.org/10.1111/jdi.12094

Kostich, W.A., Chen, A., Sperle, K., Largent, B.L., 1998. Molecular Identification and Analysis of a Novel Human Corticotropin-Releasing Factor (CRF) Receptor: The CRF2γ Receptor. Mol Endocrinol 12, 1077–1085. https://doi.org/10.1210/mend.12.8.0145

Kotani, M., Detheux, M., Vandenbogaerde, A., Communi, D., Vanderwinden, J.-M., Le Poul, E., Brézillon, S., Tyldesley, R., Suarez-Huerta, N., Vandeput, F., Blanpain, C., Schiffmann, S.N., Vassart, G., Parmentier, M., 2001. The Metastasis Suppressor Gene KiSS-1 Encodes Kisspeptins, the Natural Ligands of the Orphan G Protein-coupled Receptor GPR54. J. Biol. Chem. 276, 34631–34636. https://doi.org/10.1074/jbc.M104847200

Kowalski, G.M., Bruce, C.R., 2014. The regulation of glucose metabolism: implications and considerations for the assessment of glucose homeostasis in rodents. American Journal of Physiology-Endocrinology and Metabolism 307, E859–E871. https://doi.org/10.1152/ajpendo.00165.2014

Kramer, C.K., Campbell, S., Retnakaran, R., 2019. Gestational diabetes and the risk of cardiovascular disease in women: a systematic review and meta-analysis. Diabetologia 62, 905–914. https://doi.org/10.1007/s00125-019-4840-2

Kuperman, Y., Chen, A., 2008. Urocortins: emerging metabolic and energy homeostasis perspectives. Trends in Endocrinology & Metabolism 19, 122–129. https://doi.org/10.1016/j.tem.2007.12.002

Lacroix, M., Battista, M.-C., Doyon, M., Moreau, J., Patenaude, J., Guillemette, L., Ménard, J., Ardilouze, J.-L., Perron, P., Hivert, M.-F., 2016. Higher maternal leptin levels at second trimester are associated with subsequent greater gestational weight gain in late pregnancy. BMC Pregnancy and Childbirth 16, 62. https://doi.org/10.1186/s12884-016-0842-y

Lain, K.Y., Catalano, P.M., 2007. Metabolic Changes in Pregnancy: Clinical Obstetrics and Gynecology 50, 938–948. https://doi.org/10.1097/GRF.0b013e31815a5494

Lapatto, R., Pallais, J.C., Zhang, D., Chan, Y.-M., Mahan, A., Cerrato, F., Le, W.W., Hoffman, G.E., Seminara, S.B., 2007. Kiss1-/- mice exhibit more variable hypogonadism than Gpr54-/- mice. Endocrinology 148, 4927–4936. https://doi.org/10.1210/en.2007-0078

Larauche, M., Kiank, C., Tache, Y., 2009. Corticotropin Releasing Factor signaling in colon and ileum: regulation by stress and pathophysiological implications. J Physiol Pharmacol 60, 33–46.

Lauenborg, J., Grarup, N., Damm, P., Borch-Johnsen, K., Jørgensen, T., Pedersen, O., Hansen, T., 2009. Common Type 2 Diabetes Risk Gene Variants Associate with Gestational Diabetes. J Clin Endocrinol Metab 94, 145–150. https://doi.org/10.1210/jc.2008-1336

Lavine, R.L., Voyles, N., Perrino, P.V., Recant, L., 1977. Functional abnormalities of islets of Langerhans of obese hyperglycemic mouse. Am. J. Physiol. 233, E86-90. https://doi.org/10.1152/ajpendo.1977.233.2.E86

Layden, B.T., Durai, V., Newman, M.V., Marinelarena, A.M., Ahn, C.W., Feng, G., Lin, S., Zhang, X., Kaufman, D.B., Jafari, N., Sørensen, G.L., Lowe, W.L., 2010. Regulation of

pancreatic islet gene expression in mouse islets by pregnancy. Journal of Endocrinology 207, 265–279. https://doi.org/10.1677/JOE-10-0298

Lee, A.K., Tse, F.W., Tse, A., 2015. Arginine Vasopressin Potentiates the Stimulatory Action of CRH on Pituitary Corticotropes via a Protein Kinase C–Dependent Reduction of the Background TREK-1 Current. Endocrinology 156, 3661–3672. https://doi.org/10.1210/en.2015-1293

Lekva, T., Roland, M.C.P., Michelsen, A.E., Friis, C.M., Aukrust, P., Bollerslev, J., Henriksen, T., Ueland, T., 2017. Large Reduction in Adiponectin During Pregnancy Is Associated With Large-for-Gestational-Age Newborns. J Clin Endocrinol Metab 102, 2552–2559. https://doi.org/10.1210/jc.2017-00289

Levy, J.C., Matthews, D.R., Hermans, M.P., 1998. Correct Homeostasis Model Assessment (HOMA) Evaluation Uses the Computer Program. Diabetes Care 21, 2191–2192. https://doi.org/10.2337/diacare.21.12.2191

Lewis, K., Li, C., Perrin, M.H., Blount, A., Kunitake, K., Donaldson, C., Vaughan, J., Reyes, T.M., Gulyas, J., Fischer, W., Bilezikjian, L., Rivier, J., Sawchenko, P.E., Vale, W.W., 2001a. Identification of urocortin III, an additional member of the corticotropin-releasing factor (CRF) family with high affinity for the CRF2 receptor. Proc. Natl. Acad. Sci. U.S.A. 98, 7570–7575. https://doi.org/10.1073/pnas.121165198

Li, C., Chen, P., Vaughan, J., Blount, A., Chen, A., Jamieson, P.M., Rivier, J., Smith, M.S., Vale, W., 2003. Urocortin III Is Expressed in Pancreatic β-Cells and Stimulates Insulin and Glucagon Secretion. Endocrinology 144, 3216–3224. https://doi.org/10.1210/en.2002-0087

Li, C., Chen, P., Vaughan, J., Lee, K.-F., Vale, W., 2007. Urocortin 3 regulates glucose-stimulated insulin secretion and energy homeostasis. Proc. Natl. Acad. Sci. U.S.A. 104, 4206–4211. https://doi.org/10.1073/pnas.0611641104

Lindström, P., 2010. β-Cell Function in Obese-Hyperglycemic Mice [ob/ob Mice], in: Islam, Md.S. (Ed.), The Islets of Langerhans, Advances in Experimental Medicine and Biology. Springer Netherlands, Dordrecht, pp. 463–477. https://doi.org/10.1007/978-90-481-3271-3_20

Lovenberg, T.W., Chalmers, D.T., Liu, C., De Souza, E.B., 1995a. CRF2 alpha and CRF2 beta receptor mRNAs are differentially distributed between the rat central nervous system and peripheral tissues. Endocrinology 136, 4139–4142. https://doi.org/10.1210/endo.136.9.7544278

Lovenberg, T.W., Liaw, C.W., Grigoriadis, D.E., Clevenger, W., Chalmers, D.T., De Souza, E.B., Oltersdorf, T., 1995b. Cloning and characterization of a functionally distinct corticotropin-releasing factor receptor subtype from rat brain. Proc. Natl. Acad. Sci. U.S.A. 92, 836–840.

Lytras, N., Grossman, A., Rees, L.H., Schally, A.V., Bloom, S.R., Besser, G.M., 1984. Corticotrophin Releasing Factor: Effects on Circulating Gut and Pancreatic Peptides in Man. Clinical Endocrinology 20, 725–729. https://doi.org/10.1111/j.1365-2265.1984.tb00123.x

MacDonald, M.J., Longacre, M.J., Stoker, S.W., Kendrick, M., Thonpho, A., Brown, L.J., Hasan, N.M., Jitrapakdee, S., Fukao, T., Hanson, M.S., Fernandez, L.A., Odorico, J., 2011. Differences between Human and Rodent Pancreatic Islets. J Biol Chem 286, 18383–18396. https://doi.org/10.1074/jbc.M111.241182

MacDonald, P.E., El-kholy, W., Riedel, M.J., Salapatek, A.M.F., Light, P.E., Wheeler, M.B., 2002. The Multiple Actions of GLP-1 on the Process of Glucose-Stimulated Insulin Secretion. Diabetes 51, S434–S442. https://doi.org/10.2337/diabetes.51.2007.S434

Mahler, R.J., Adler, M.L., 1999. Type 2 Diabetes Mellitus: Update on Diagnosis, Pathophysiology, and Treatment. J Clin Endocrinol Metab 84, 1165–1171. https://doi.org/10.1210/jcem.84.4.5612

Markovic, D., Punn, A., Lehnert, H., Grammatopoulos, D.K., 2011. Molecular determinants and feedback circuits regulating type 2 CRH receptor signal integration. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, Including the Special Section: 11th European Symposium on Calcium 1813, 896–907. https://doi.org/10.1016/j.bbamcr.2011.02.005

Marzorati, S., Ramirez-Dominguez, M., 2015. Mouse Islet Isolation, in: Islam, Md.S. (Ed.), Islets of Langerhans. Springer Netherlands, Dordrecht, pp. 83–107. https://doi.org/10.1007/978-94-007-6686-0_33

Matthews, D.R., Hosker, J.P., Rudenski, A.S., Naylor, B.A., Treacher, D.F., Turner, R.C., 1985. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 28, 412–419. https://doi.org/10.1007/BF00280883

Mazaki-Tovi, S., Kanety, H., Pariente, C., Hemi, R., Yissachar, E., Schiff, E., Cohen, O., Sivan, E., 2011. Insulin sensitivity in late gestation and early postpartum period: the role of circulating maternal adipokines. Gynecological Endocrinology 27, 725–731. https://doi.org/10.3109/09513590.2010.500426

McCulloch, L.J., van de Bunt, M., Braun, M., Frayn, K.N., Clark, A., Gloyn, A.L., 2011. GLUT2 (SLC2A2) is not the principal glucose transporter in human pancreatic beta cells: implications for understanding genetic association signals at this locus. Mol. Genet. Metab. 104, 648–653. https://doi.org/10.1016/j.ymgme.2011.08.026

McIntyre, H.D., Catalano, P., Zhang, C., Desoye, G., Mathiesen, E.R., Damm, P., 2019. Gestational diabetes mellitus. Nat Rev Dis Primers 5, 1–19. https://doi.org/10.1038/s41572-019-0098-8

McLean, M., Bisits, A., Davies, J., Woods, R., Lowry, P., Smith, R., 1995. A placental clock controlling the length of human pregnancy. Nature Medicine 1, 460–463. https://doi.org/10.1038/nm0595-460

McLean, M., Smith, R., 1999. Corticotropin-releasing Hormone in Human Pregnancy and Parturition. Trends in Endocrinology & Metabolism 10, 174–178. https://doi.org/10.1016/S1043-2760(98)00146-5

Metzger, B.E., 1991. Summary and Recommendations of the Third International Workshop-Conference on Gestational Diabetes Mellitus. Diabetes 40, 197–201. https://doi.org/10.2337/diab.40.2.S197

Milner, R.D.G., Hales, C.N., 1967. The role of calcium and magnesium in insulin secretion from rabbit pancreas studied in vitro. Diabetologia 3, 47–49. https://doi.org/10.1007/BF01269910

Misra, V.K., Trudeau, S., 2011. The influence of overweight and obesity on longitudinal trends in maternal serum leptin levels during pregnancy. Obesity (Silver Spring, Md.) 19, 416–421. https://doi.org/10.1038/oby.2010.172

Moltz, J.H., Fawcett, C.P., 1985a. Corticotropin-releasing factor: its action on the islets of Langerhans. Endocr. Res. 11, 87–93. https://doi.org/10.3109/07435808509035427

Moltz, J.H., Fawcett, C.P., 1985b. Corticotropin-releasing factor inhibits insulin release from perfused rat pancreas. Am. J. Physiol. 248, E741-743. https://doi.org/10.1152/ajpendo.1985.248.6.E741

Moore, L.E., Clokey, D., Rappaport, V.J., Curet, L.B., 2010. Metformin Compared With Glyburide in Gestational Diabetes: A Randomized Controlled Trial. Obstetrics & Gynecology 115, 55. https://doi.org/10.1097/AOG.0b013e3181c52132

Moritoh, Y., Takeuchi, K., Asakawa, T., Kataoka, O., Odaka, H., 2008. Chronic administration of alogliptin, a novel, potent, and highly selective dipeptidyl peptidase-4 inhibitor, improves glycemic control and beta-cell function in obese diabetic ob/ob mice. Eur. J. Pharmacol. 588, 325–332. https://doi.org/10.1016/j.ejphar.2008.04.018

Moskalewski, S., 1965. Isolation and culture of the islets of Langerhans of the guinea pig. General and Comparative Endocrinology 5, 342–353. https://doi.org/10.1016/0016-6480(65)90059-6

Moss, A.C., Anton, P., Savidge, T., Newman, P., Cheifetz, A.S., Gay, J., Paraschos, S., Winter, M.W., Moyer, M.P., Karalis, K., Kokkotou, E., Pothoulakis, C., 2007. Urocortin II mediates pro-inflammatory effects in human colonocytes via corticotropin-releasing hormone receptor 2α. Gut 56, 1210–1217. https://doi.org/10.1136/gut.2006.110668

Moyce, B.L., Dolinsky, V.W., 2018. Maternal β-Cell Adaptations in Pregnancy and Placental Signalling: Implications for Gestational Diabetes. Int J Mol Sci 19. https://doi.org/10.3390/ijms19113467

Muglia, L., Jacobson, L., Dikkes, P., Majzoub, J.A., 1995. Corticotropin-releasing hormone deficiency reveals major fetal but not adult glucocorticoid need. Nature 373, 427–432. https://doi.org/10.1038/373427a0

Mumtaz, A., Khalid, A., Jamil, Z., Fatima, S.S., Arif, S., Rehman, R., 2017. Kisspeptin: A Potential Factor for Unexplained Infertility and Impaired Embryo Implantation. Int J Fertil Steril 11, 99–104. https://doi.org/10.22074/ijfs.2017.4957

Nadal, A., Alonso-Magdalena, P., Soriano, S., Ropero, A.B., Quesada, I., 2009. The role of oestrogens in the adaptation of islets to insulin resistance. The Journal of Physiology 587, 5031. https://doi.org/10.1113/jphysiol.2009.177188

Nahavandi, S., Price, S., Sumithran, P., Ekinci, E.I., 2019. Exploration of the shared pathophysiological mechanisms of gestational diabetes and large for gestational age offspring. World J Diabetes 10, 333–340. https://doi.org/10.4239/wjd.v10.i6.333

Napso, T., Yong, H.E.J., Lopez-Tello, J., Sferruzzi-Perri, A.N., 2018. The Role of Placental Hormones in Mediating Maternal Adaptations to Support Pregnancy and Lactation. Front. Physiol. 9. https://doi.org/10.3389/fphys.2018.01091

Nemoto, T., Mano-Otagiri, A., Shibasaki, T., 2005. Urocortin 2 induces tyrosine hydroxylase phosphorylation in PC12 cells. Biochemical and Biophysical Research Communications 330, 821–831. https://doi.org/10.1016/j.bbrc.2005.03.031

Neufeld-Cohen, A., Tsoory, M.M., Evans, A.K., Getselter, D., Gil, S., Lowry, C.A., Vale, W.W., Chen, A., 2010. A triple urocortin knockout mouse model reveals an essential role for urocortins in stress recovery. PNAS 107, 19020–19025. https://doi.org/10.1073/pnas.1013761107

Neuman, J.C., Truchan, N.A., Joseph, J.W., Kimple, M.E., 2014. A Method for Mouse Pancreatic Islet Isolation and Intracellular cAMP Determination. J Vis Exp. https://doi.org/10.3791/50374

Newbern, D., Freemark, M., 2011. Placental hormones and the control of maternal metabolism and fetal growth. Curr Opin Endocrinol Diabetes Obes 18, 409–416. https://doi.org/10.1097/MED.0b013e32834c800d

Newsholme, P., Brennan, L., Bender, K., 2006. Amino Acid Metabolism, β-Cell Function, and Diabetes. Diabetes 55, S39–S47. https://doi.org/10.2337/db06-S006

Newsholme, P., Krause, M., 2012. Nutritional Regulation of Insulin Secretion: Implications for Diabetes. Clin Biochem Rev 33, 35–47.

Nguyen-Ngo, C., Jayabalan, N., Salomon, C., Lappas, M., 2019. Molecular pathways disrupted by gestational diabetes mellitus. Journal of Molecular Endocrinology 63, R51–R72. https://doi.org/10.1530/JME-18-0274

NICE-Diabetes in pregnancy, 2015. Diabetes in pregnancy: management from preconception to the postnatal period | Guidance | NICE [WWW Document]. URL https://www.nice.org.uk/guidance/ng3/chapter/1-Recommendations#gestational-diabetes-2 (accessed 1.3.20).

Nolan, A.L., O'Dowd, J.F., 2009. The Measurement of Insulin Secretion from Isolated Rodent Islets of Langerhans, in: Stocker, C. (Ed.), Type 2 Diabetes: Methods and Protocols, Methods in Molecular Biology. Humana Press, Totowa, NJ, pp. 43–51. https://doi.org/10.1007/978-1-59745-448-3_4

Nolan, C.J., Damm, P., Prentki, M., 2011. Type 2 diabetes across generations: from pathophysiology to prevention and management. The Lancet 378, 169–181. https://doi.org/10.1016/S0140-6736(11)60614-4

Novembri, R., De Clemente, C., Funghi, L., Torricelli, M., Voltolini, C., Challis, J.R., Petraglia, F., 2015. Corticotropin releasing hormone and Urocortin 2 activate inflammatory pathways in cultured trophoblast cell lines. European Journal of Obstetrics & Gynecology and Reproductive Biology 195, 200–205. https://doi.org/10.1016/j.ejogrb.2015.10.027

O'Carroll, A.-M., Howell, G.M., Roberts, E.M., Lolait, S.J., 2008. Vasopressin potentiates corticotropin-releasing hormone-induced insulin release from mouse pancreatic β-cells. J Endocrinol 197, 231–239. https://doi.org/10.1677/JOE-07-0645

Oh, C.-M., Park, S., Kim, H., 2016. Serotonin as a New Therapeutic Target for Diabetes Mellitus and Obesity. Diabetes Metab J 40, 89–98. https://doi.org/10.4093/dmj.2016.40.2.89

Oh, D.Y., Olefsky, J.M., 2016. G protein-coupled receptors as targets for anti-diabetic therapeutics. Nature Reviews Drug Discovery 15, 161–172. https://doi.org/10.1038/nrd.2015.4

Ohara-Imaizumi, M., Kim, H., Yoshida, M., Fujiwara, T., Aoyagi, K., Toyofuku, Y., Nakamichi, Y., Nishiwaki, C., Okamura, T., Uchida, T., Fujitani, Y., Akagawa, K., Kakei, M., Watada, H., German, M.S., Nagamatsu, S., 2013. Serotonin regulates glucose-stimulated insulin secretion from pancreatic β cells during pregnancy. Proc. Natl. Acad. Sci. U.S.A. 110, 19420–19425. https://doi.org/10.1073/pnas.1310953110

O'Harte, F.P., Mooney, M.H., Kelly, C.M., Flatt, P.R., 2000. Improved glycaemic control in obese diabetic ob/ob mice using N-terminally modified gastric inhibitory polypeptide. J. Endocrinol. 165, 639–648. https://doi.org/10.1677/joe.0.1650639

Ohtaki, T., Shintani, Y., Honda, S., Matsumoto, H., Hori, A., Kanehashi, K., Terao, Y., Kumano, S., Takatsu, Y., Masuda, Y., Ishibashi, Y., Watanabe, T., Asada, M., Yamada, T., Suenaga, M., Kitada, C., Usuki, S., Kurokawa, T., Onda, H., Nishimura, O., Fujino, M., 2001. Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor. Nature 411, 613–617. https://doi.org/10.1038/35079135

Okamoto, M., Ohara-Imaizumi, M., Kubota, N., Hashimoto, S., Eto, K., Kanno, T., Kubota, T., Wakui, M., Nagai, R., Noda, M., Nagamatsu, S., Kadowaki, T., 2008. Adiponectin induces insulin secretion in vitro and in vivo at a low glucose concentration. Diabetologia 51, 827–835. https://doi.org/10.1007/s00125-008-0944-9

Okuno, S., Akazawa, S., Yasuhi, I., Kawasaki, E., Matsumoto, K., Yamasaki, H., Matsuo, H., Yamaguchi, Y., Nagataki, S., 1995. Decreased expression of the GLUT4 glucose transporter protein in adipose tissue during pregnancy. Horm. Metab. Res. 27, 231–234. https://doi.org/10.1055/s-2007-979946

Orci, L., Malaisse-Lagae, F., Ravazzola, M., Rouiller, D., Renold, A.E., Perrelet, A., Unger, R., 1975. A morphological basis for intercellular communication between alpha- and beta-cells in the endocrine pancreas. J Clin Invest 56, 1066–1070.

Orth, D.N., Mount, C.D., 1987. Specific high-affinity binding protein for human corticotropin-releasing hormone in normal human plasma. Biochemical and Biophysical Research Communications 143, 411–417. https://doi.org/10.1016/0006-291X(87)91369-6

Ouchi, N., Parker, J.L., Lugus, J.J., Walsh, K., 2011. Adipokines in inflammation and metabolic disease. Nat Rev Immunol 11, 85–97. https://doi.org/10.1038/nri2921

Pacini, G., Mari, A., 2003. Methods for clinical assessment of insulin sensitivity and β-cell function. Best Practice & Research Clinical Endocrinology & Metabolism, Carbohydrate Metabolism 17, 305–322. https://doi.org/10.1016/S1521-690X(03)00042-3

Pala, H.G., Ozalp, Y., Yener, A.S., Gerceklioglu, G., Uysal, S., Onvural, A., 2015. Adiponectin Levels in Gestational Diabetes Mellitus and in Pregnant Women Without Glucose Intolerance. Advances in Clinical and Experimental Medicine 24, 85–92. https://doi.org/10.17219/acem/38141

Park, K.S., 2011. The Search for Genetic Risk Factors of Type 2 Diabetes Mellitus. Diabetes Metab J 35, 12–22. https://doi.org/10.4093/dmj.2011.35.1.12

Parsons, J.A., Brelje, T.C., Sorenson, R.L., 1992. Adaptation of islets of Langerhans to pregnancy: increased islet cell proliferation and insulin secretion correlates with the onset of placental lactogen secretion. Endocrinology 130, 1459–1466. https://doi.org/10.1210/endo.130.3.1537300

Paruthiyil, S., Hagiwara, S., Kundassery, K., Bhargava, A., 2018. Sexually dimorphic metabolic responses mediated by CRF2 receptor during nutritional stress in mice. Biol Sex Differ 9, 49. https://doi.org/10.1186/s13293-018-0208-4

Paschos, K., Chouridou, E., Koureta, M., Lambropoulou, M., Kolios, G., Chatzaki, E., 2013. The corticotropin releasing factor system in the liver: expression, actions and possible implications in hepatic physiology and pathology. HORMONES 12, 236–245. https://doi.org/10.14310/horm.2002.1407

Paschos, K.A., Kolios, G., Chatzaki, E., 2009. The corticotropin-releasing factor system in inflammatory bowel disease: Prospects for new therapeutic approaches. Drug Discovery Today 14, 713–720. https://doi.org/10.1016/j.drudis.2009.04.002

Pasek, R.C., Gannon, M., 2013. Advancements and challenges in generating accurate animal models of gestational diabetes mellitus. Am. J. Physiol. Endocrinol. Metab. 305, E1327-1338. https://doi.org/10.1152/ajpendo.00425.2013

Patel, K., Rademaker, M., Kirkpatrick, C., Charles, C., Fisher, S., Yandle, T., Richards, A., 2012. Comparative pharmacokinetics and pharmacodynamics of urocortins 1, 2 and 3 in healthy sheep. Br J Pharmacol 166, 1916–1925. https://doi.org/10.1111/j.1476-5381.2012.01904.x

Pedersen, S.D., 2013. Metabolic complications of obesity. Best Practice & Research Clinical Endocrinology & Metabolism, Complications of Obesity 27, 179–193. https://doi.org/10.1016/j.beem.2013.02.004

Pendergrass, M., Fazioni, E., DeFronzo, R.A., 1995. Non-insulin-dependent diabetes mellitus and gestational diabetes mellitus: Same disease, another name? Diabetes Reviews 3, 566–583.

Pepels, P.P.L.M., Spaanderman, M.E.A., Hermus, A.R.M.M., Lotgering, F.K., Sweep, C.G.J., 2010. Placental urocortin-2 and -3: endocrine or paracrine functioning during healthy pregnancy? Placenta 31, 475–481. https://doi.org/10.1016/j.placenta.2010.03.012

Perkins, A.V., Linton, E.A., 1995. Placental corticotrophin-releasing hormone: there by accident or design? Journal of Endocrinology 147, 377–381. https://doi.org/10.1677/joe.0.1470377

Persaud, S.J., 2017. Islet G-protein coupled receptors: therapeutic potential for diabetes. Current Opinion in Pharmacology, • Gastrointestinal • Endocrine and metabolic diseases 37, 24–28. https://doi.org/10.1016/j.coph.2017.08.001

Persaud, S.J., Asare-Anane, H., Jones, P.M., 2002. Insulin receptor activation inhibits insulin secretion from human islets of Langerhans. FEBS Letters 510, 225–228. https://doi.org/10.1016/S0014-5793(01)03268-9

Petersen, M.C., Shulman, G.I., 2018. Mechanisms of Insulin Action and Insulin Resistance. Physiol Rev 98, 2133–2223. https://doi.org/10.1152/physrev.00063.2017

Petraglia, F., Imperatore, A., Challis, J.R.G., 2010. Neuroendocrine Mechanisms in Pregnancy and Parturition. Endocrine Reviews 31, 783–816. https://doi.org/10.1210/er.2009-0019

Petraglia, F., Sawchenko, P.E., Rivier, J., Vale, W., 1987. Evidence for local stimulation of ACTH secretion by corticotropin-releasing factor in human placenta. Nature 328, 717–719. https://doi.org/10.1038/328717a0

Petrusz, P., Merchenthaler, I., Maderdrut, J.L., Vigh, S., Schally, A.V., 1983. Corticotropin-releasing factor (CRF)-like immunoreactivity in the vertebrate endocrine pancreas. PNAS 80, 1721–1725. https://doi.org/10.1073/pnas.80.6.1721

Peyot, M.-L., Gray, J.P., Lamontagne, J., Smith, P.J.S., Holz, G.G., Madiraju, S.R.M., Prentki, M., Heart, E., 2009. Glucagon-like peptide-1 induced signaling and insulin secretion do not drive fuel and energy metabolism in primary rodent pancreatic beta-cells. PLoS One 4, e6221. https://doi.org/10.1371/journal.pone.0006221

Plows, J.F., Stanley, J.L., Baker, P.N., Reynolds, C.M., Vickers, M.H., 2018. The Pathophysiology of Gestational Diabetes Mellitus. Int J Mol Sci 19. https://doi.org/10.3390/ijms19113342

Poolsup, N., Suksomboon, N., Amin, M., 2014. Efficacy and safety of oral antidiabetic drugs in comparison to insulin in treating gestational diabetes mellitus: a meta-analysis. PLoS ONE 9, e109985. https://doi.org/10.1371/journal.pone.0109985

Powe, C.E., Allard, C., Battista, M.-C., Doyon, M., Bouchard, L., Ecker, J.L., Perron, P., Florez, J.C., Thadhani, R., Hivert, M.-F., 2016. Heterogeneous Contribution of Insulin Sensitivity and Secretion Defects to Gestational Diabetes Mellitus. Diabetes Care 39, 1052–1055. https://doi.org/10.2337/dc15-2672

Powe, C.E., Huston Presley, L.P., Locascio, J.J., Catalano, P.M., 2019. Augmented insulin secretory response in early pregnancy. Diabetologia. https://doi.org/10.1007/s00125-019-4881-6

Power, M., Williams, L., Gibson, S., Schulkin, J., Helfers, J., Zorrilla, E., 2010. Pattern of maternal circulating CRH in laboratory-housed squirrel and owl monkeys. Am J Primatol 72, 1004–1012. https://doi.org/10.1002/ajp.20850

Power, M.L., Bowman, M.E., Smith, R., Ziegler, T.E., Layne, D.G., Schulkin, J., Tardif, S.D., 2006. Pattern of maternal serum corticotropin-releasing hormone concentration during

pregnancy in the common marmoset (Callithrix jacchus). American Journal of Primatology 68, 181–188. https://doi.org/10.1002/ajp.20215
Power, M.L., Schulkin, J., 2006. Functions of corticotropin-releasing hormone in anthropoid primates: From brain to placenta. American Journal of Human Biology 18, 431–447. https://doi.org/10.1002/ajhb.20521
Proks, P., Ashcroft, F.M., 1995. Effects of divalent cations on exocytosis and endocytosis from single mouse pancreatic beta-cells. The Journal of Physiology 487, 465–477. https://doi.org/10.1113/jphysiol.1995.sp020893
Qiao, L., Saget, S., Lu, C., Hay, W.W., Karsenty, G., Shao, J., 2020. Adiponectin Promotes Maternal β-Cell Expansion Through Placental Lactogen Expression. Diabetes. https://doi.org/10.2337/db20-0471
Qiao, L., Wattez, J.-S., Lee, S., Nguyen, A., Schaack, J., Hay, W.W., Shao, J., 2017. Adiponectin Deficiency Impairs Maternal Metabolic Adaptation to Pregnancy in Mice. Diabetes 66, 1126–1135. https://doi.org/10.2337/db16-1096
Qiu, C., Williams, M.A., Vadachkoria, S., Frederick, I.O., Luthy, D.A., 2004. Increased maternal plasma leptin in early pregnancy and risk of gestational diabetes mellitus. Obstetrics and Gynecology 103, 519–525. https://doi.org/10.1097/01.AOG.0000113621.53602.7a
R J, R., K E, H., M F, H., 2014. Gestational diabetes mellitus management with oral hypoglycemic agents. Semin Perinatol 38, 508–515. https://doi.org/10.1053/j.semperi.2014.08.012
Rademaker Miriam T., Cameron Vicky A., Charles Christopher J., Richards A. Mark, 2005. Integrated Hemodynamic, Hormonal, and Renal Actions of Urocortin 2 in Normal and Paced Sheep. Circulation 112, 3624–3632. https://doi.org/10.1161/CIRCULATIONAHA.105.561308
Rademaker Miriam T., Charles Christopher J., Ellmers Leigh J., Lewis Lynley K., Nicholls M. Gary, Richards A. Mark, 2011. Prolonged Urocortin 2 Administration in Experimental Heart Failure. Hypertension 57, 1136–1144. https://doi.org/10.1161/HYPERTENSIONAHA.111.173203
Raff, H., Sharma, S.T., Nieman, L.K., 2014. Physiological Basis for the Etiology, Diagnosis, and Treatment of Adrenal Disorders: Cushing's Syndrome, Adrenal Insufficiency, and Congenital Adrenal Hyperplasia. Compr Physiol 4, 739–769. https://doi.org/10.1002/cphy.c130035
Ramírez-Domínguez, M., 2016. Historical Background of Pancreatic Islet Isolation, in: Ramírez-Domínguez, M. (Ed.), Pancreatic Islet Isolation: From the Mouse to the Clinic, Advances in Experimental Medicine and Biology. Springer International Publishing, Cham, pp. 1–9. https://doi.org/10.1007/978-3-319-39824-2_1
Ramos-Payán, R., Aguilar-Medina, M., Estrada-Parra, S., González-y-Merchand, J.A., Favila-Castillo, L., Monroy-Ostria, A., Estrada-Garcia, I.C.E., 2003. Quantification of Cytokine Gene Expression Using an Economical Real-Time Polymerase Chain Reaction Method Based on SYBR® Green I. Scandinavian Journal of Immunology 57, 439–445. https://doi.org/10.1046/j.1365-3083.2003.01250.x
Rassouli, O., Liapakis, G., Lazaridis, I., Sakellaris, G., Gkountelias, K., Gravanis, A., Margioris, A.N., Karalis, K.P., Venihaki, M., 2011. A Novel Role of Peripheral Corticotropin-Releasing Hormone (CRH) on Dermal Fibroblasts. PLOS ONE 6, e21654. https://doi.org/10.1371/journal.pone.0021654
Rassouli, O., Liapakis, G., Venihaki, M., 2018. Role of Central and Peripheral CRH in Skin. Curr Mol Pharmacol 11, 72–80. https://doi.org/10.2174/1874467209666161026144219
Ratajczak, C.K., Muglia, L.J., 2008. Insights Into Parturition Biology From Genetically Altered Mice. Pediatric Research 64, 581–589. https://doi.org/10.1203/PDR.0b013e31818718d2
Rebouças, E. de L., Costa, J.J. do N., Passos, M.J., Passos, J.R. de S., Hurk, R. van den, Silva, J.R.V., 2013. Real time PCR and importance of housekeepings genes for normalization and quantification of mRNA expression in different tissues. Brazilian Archives of Biology and Technology 56, 143–154. https://doi.org/10.1590/S1516-89132013000100019
Reece, E.A., 2010. The fetal and maternal consequences of gestational diabetes mellitus. The Journal of Maternal-Fetal & Neonatal Medicine 23, 199–203. https://doi.org/10.3109/14767050903550659

Rendell, M., 2004. The role of sulphonylureas in the management of type 2 diabetes mellitus. Drugs 64, 1339–1358. https://doi.org/10.2165/00003495-200464120-00006
Retnakaran, R., 2017. Adiponectin and β-Cell Adaptation in Pregnancy. Diabetes 66, 1121–1122. https://doi.org/10.2337/dbi17-0001
Retnakaran, R., Ye, C., Kramer, C.K., Connelly, P.W., Hanley, A.J., Sermer, M., Zinman, B., 2016. Evaluation of Circulating Determinants of Beta-Cell Function in Women With and Without Gestational Diabetes. The Journal of Clinical Endocrinology and Metabolism 101, 2683–2691. https://doi.org/10.1210/jc.2016-1402
Reul, J.M.H.M., Holsboer, F., 2002. On the role of corticotropin-releasing hormone receptors in anxiety and depression. Dialogues Clin Neurosci 4, 31–46.
Reyes, T.M., Lewis, K., Perrin, M.H., Kunitake, K.S., Vaughan, J., Arias, C.A., Hogenesch, J.B., Gulyas, J., Rivier, J., Vale, W.W., Sawchenko, P.E., 2001. Urocortin II: A member of the corticotropin-releasing factor (CRF) neuropeptide family that is selectively bound by type 2 CRF receptors. PNAS 98, 2843–2848. https://doi.org/10.1073/pnas.051626398
Rieck, S., Kaestner, K.H., 2010. Expansion of beta-cell mass in response to pregnancy. Trends Endocrinol. Metab. 21, 151–158. https://doi.org/10.1016/j.tem.2009.11.001
Rieck, S., White, P., Schug, J., Fox, A.J., Smirnova, O., Gao, N., Gupta, R.K., Wang, Z.V., Scherer, P.E., Keller, M.P., Attie, A.D., Kaestner, K.H., 2009. The Transcriptional Response of the Islet to Pregnancy in Mice. Mol Endocrinol 23, 1702–1712. https://doi.org/10.1210/me.2009-0144
Risbrough, V.B., Stein, M.B., 2006. Role of Corticotropin Releasing Factor in Anxiety Disorders: A Translational Research Perspective. Horm Behav 50, 550–561. https://doi.org/10.1016/j.yhbeh.2006.06.019
Rivier, J.E., Rivier, C.L., 2014. Corticotropin-releasing factor peptide antagonists: design, characterization and potential clinical relevance. Front Neuroendocrinol 35, 161–170. https://doi.org/10.1016/j.yfrne.2013.10.006
Robinson, B.G., Arbiser, J.L., Emanuel, R.L., Majzoub, J.A., 1989. Species-specific placental corticotropin releasing hormone messenger RNA and peptide expression. Molecular and Cellular Endocrinology 62, 337–341. https://doi.org/10.1016/0303-7207(89)90022-1
Röder, Pia V, Wu, B., Liu, Y., Han, W., 2016. Pancreatic regulation of glucose homeostasis. Exp Mol Med 48, e219–e219. https://doi.org/10.1038/emm.2016.6
Rodiño-Janeiro, B.K., Alonso-Cotoner, C., Pigrau, M., Lobo, B., Vicario, M., Santos, J., 2015. Role of Corticotropin-releasing Factor in Gastrointestinal Permeability. J Neurogastroenterol Motil 21, 33–50. https://doi.org/10.5056/jnm14084
Rodrigo, N., Glastras, S.J., 2020. Pathophysiology Underpinning Gestational Diabetes Mellitus and the Role of Biomarkers for its Prediction. EMJ Diabet. https://doi.org/10.33590/emj/19-00150
Roglic, G., World Health Organization (Eds.), 2016. Global report on diabetes. World Health Organization, Geneva, Switzerland.
Rolin, B., Larsen, M.O., Gotfredsen, C.F., Deacon, C.F., Carr, R.D., Wilken, M., Knudsen, L.B., 2002. The long-acting GLP-1 derivative NN2211 ameliorates glycemia and increases beta-cell mass in diabetic mice. Am. J. Physiol. Endocrinol. Metab. 283, E745-752. https://doi.org/10.1152/ajpendo.00030.2002
Rorsman, P., Huising, M.O., 2018. The somatostatin-secreting pancreatic δ-cell in health and disease. Nat Rev Endocrinol 14, 404–414. https://doi.org/10.1038/s41574-018-0020-6
Rosen, T., Schulkin, J., Power, M., Tadesse, S., Norwitz, E.R., Wen, Z., Wang, B., 2015. Comparative Immunohistochemistry of Placental Corticotropin-Releasing Hormone and the Transcription Factor RelB–NFκB2 Between Humans and Nonhuman Primates. Comp Med 65, 140–143.
Rosenbaum, D.M., Rasmussen, S.G.F., Kobilka, B.K., 2009. The structure and function of G-protein-coupled receptors. Nature 459, 356–363. https://doi.org/10.1038/nature08144
Roustit, M.M., Vaughan, J.M., Jamieson, P.M., Cleasby, M.E., 2014. Urocortin 3 activates AMPK and AKT pathways and enhances glucose disposal in rat skeletal muscle. J Endocrinol 223, 143–154. https://doi.org/10.1530/JOE-14-0181
Ryabinin, A.E., Tsoory, M.M., Kozicz, T., Thiele, T.E., Neufeld – Cohen, A., Chen, A., Lowery, E.G., Giardino, W.J., Kaur, S., 2012. Urocortins: CRF's siblings and their potential role

in anxiety, depression and alcohol drinking behavior. Alcohol 46, 349–357. https://doi.org/10.1016/j.alcohol.2011.10.007
Ryan, E.A., Enns, L., 1988. Role of Gestational Hormones in the Induction of Insulin Resistance. J Clin Endocrinol Metab 67, 341–347. https://doi.org/10.1210/jcem-67-2-341
Saeedi, P., Petersohn, I., Salpea, P., Malanda, B., Karuranga, S., Unwin, N., Colagiuri, S., Guariguata, L., Motala, A.A., Ogurtsova, K., Shaw, J.E., Bright, D., Williams, R., 2019. Global and regional diabetes prevalence estimates for 2019 and projections for 2030 and 2045: Results from the International Diabetes Federation Diabetes Atlas, 9th edition. Diabetes Research and Clinical Practice 157. https://doi.org/10.1016/j.diabres.2019.107843
Saffran, M., Schally, A.V., Benfey, B.G., 1955. Stimulation of the release of corticotropin from the adenohypophysis by a neurohypophysial factor. Endocrinology 57, 439–444. https://doi.org/10.1210/endo-57-4-439
Saijonmaa, O., Laatikainen, T., Wahlström, T., 1988. Corticotrophin-releasing factor in human placenta: Localization, concentration and release in vitro. Placenta 9, 373–385. https://doi.org/10.1016/0143-4004(88)90050-1
Sakamoto, R., Matsubara, E., Nomura, M., Wang, L., Kawahara, Y., Yanase, T., Nawata, H., Takayanagi, R., 2013. Roles for corticotropin-releasing factor receptor type 1 in energy homeostasis in mice. Metabolism 62, 1739–1748. https://doi.org/10.1016/j.metabol.2013.08.005
Saphier, P.W., Faria, M., Grossman, A., Coy, D.H., Besser, G.M., Hodson, B., Parkes, M., Linton, E.A., Lowry, P.J., 1992. A comparison of the clearance of ovine and human corticotrophin-releasing hormone (CRH) in man and sheep: a possible role for CRH-binding protein. Journal of Endocrinology 133, 487–495. https://doi.org/10.1677/joe.0.1330487
Saranya, N., Suganthi, M., Shanthi, D., Navina, N., 2018. Comparison of one step glucose tolerance test (75 g GTT) and two step glucose tolerance test (100 g GTT) in screening and diagnosis of gestational diabetes mellitus. International Journal of Reproduction, Contraception, Obstetrics and Gynecology 7, 4814–4818. https://doi.org/10.18203/2320-1770.ijrcog20184691
Sasaki, A., Shinkawa, O., Margioris, A.N., Liotta, A.S., Sato, S., Murakami, O., Go, M., Shimizu, Y., Hanew, K., Yoshinaga, K., 1987. Immunoreactive corticotropin-releasing hormone in human plasma during pregnancy, labor, and delivery. J. Clin. Endocrinol. Metab. 64, 224–229. https://doi.org/10.1210/jcem-64-2-224
Sasaki, A., Tempst, P., Liotta, A.S., Margioris, A.N., Hood, L.E., Kent, S.B.H., Sato, S., Shinkawa, O., Yoshinaga, K., Krieger, D.T., 1988. Isolation and Characterization of a Corticotropin-Releasing Hormone-Like Peptide From Human Placenta. J Clin Endocrinol Metab 67, 768–773. https://doi.org/10.1210/jcem-67-4-768
Scheen, A.J., 2003. Pathophysiology of Type 2 Diabetes. Acta Clinica Belgica 58, 335–341. https://doi.org/10.1179/acb.2003.58.6.001
Schmid, J., Ludwig, B., Schally, A.V., Steffen, A., Ziegler, C.G., Block, N.L., Koutmani, Y., Brendel, M.D., Karalis, K.P., Simeonovic, C.J., Licinio, J., Ehrhart-Bornstein, M., Bornstein, S.R., 2011. Modulation of pancreatic islets-stress axis by hypothalamic releasing hormones and 11β-hydroxysteroid dehydrogenase. PNAS 108, 13722–13727. https://doi.org/10.1073/pnas.1110965108
Schraenen, A., Lemaire, K., de Faudeur, G., Hendrickx, N., Granvik, M., Van Lommel, L., Mallet, J., Vodjdani, G., Gilon, P., Binart, N., in't Veld, P., Schuit, F., 2010. Placental lactogens induce serotonin biosynthesis in a subset of mouse beta cells during pregnancy. Diabetologia 53, 2589–2599. https://doi.org/10.1007/s00125-010-1913-7
Schwetz, T.A., Reissaus, C.A., Piston, D.W., 2014. Differential Stimulation of Insulin Secretion by GLP-1 and Kisspeptin-10. PLoS One 9. https://doi.org/10.1371/journal.pone.0113020
Seasholtz, A.F., Bourbonais, F.J., Harnden, C.E., Camper, S.A., 1991. Nucleotide sequence and expression of the mouse corticotropin-releasing hormone gene. Molecular and Cellular Neuroscience 2, 266–273. https://doi.org/10.1016/1044-7431(91)90054-R
Seasholtz, A.F., Valverde, R.A., Denver, R.J., 2002. Corticotropin-releasing hormone-binding protein: biochemistry and function from fishes to mammals. Journal of Endocrinology 175, 89–97. https://doi.org/10.1677/joe.0.1750089

Seely, E.W., Solomon, C.G., 2003. Insulin Resistance and Its Potential Role in Pregnancy-Induced Hypertension. J Clin Endocrinol Metab 88, 2393–2398. https://doi.org/10.1210/jc.2003-030241

Seminara, S.B., Messager, S., Chatzidaki, E.E., Thresher, R.R., Acierno, J.S., Shagoury, J.K., Bo-Abbas, Y., Kuohung, W., Schwinof, K.M., Hendrick, A.G., Zahn, D., Dixon, J., Kaiser, U.B., Slaugenhaupt, S.A., Gusella, J.F., O'Rahilly, S., Carlton, M.B.L., Crowley, W.F., Aparicio, S.A.J.R., Colledge, W.H., 2003. The GPR54 gene as a regulator of puberty. N. Engl. J. Med. 349, 1614–1627. https://doi.org/10.1056/NEJMoa035322

Seres, J., Bornstein, S.R., Seres, P., Willenberg, H.S., Schulte, K.M., Scherbaum, W.A., Ehrhart-Bornstein, M., 2004. Corticotropin-Releasing Hormone System in Human Adipose Tissue. None 89, 965–970. https://doi.org/10.1210/jc.2003-031299

Seymour, P.A., Schmidt, A.W., Schulz, D.W., 2003. The pharmacology of CP-154,526, a non-peptide antagonist of the CRH1 receptor: a review. CNS Drug Rev 9, 57–96. https://doi.org/10.1111/j.1527-3458.2003.tb00244.x

Shanik, M.H., Xu, Y., Škrha, J., Dankner, R., Zick, Y., Roth, J., 2008. Insulin Resistance and Hyperinsulinemia: Is hyperinsulinemia the cart or the horse? Diabetes Care 31, S262–S268. https://doi.org/10.2337/dc08-s264

Shao, J., Yamashita, H., Qiao, L., Draznin, B., Friedman, J.E., 2002. Phosphatidylinositol 3-Kinase Redistribution Is Associated With Skeletal Muscle Insulin Resistance in Gestational Diabetes Mellitus. Diabetes 51, 19–29. https://doi.org/10.2337/diabetes.51.1.19

Shibasaki, T., Odagiri, E., Shizume, K., Ling, N., 1982. Corticotropin-releasing factor-like activity in human placental extracts. J. Clin. Endocrinol. Metab. 55, 384–386. https://doi.org/10.1210/jcem-55-2-384

Shirakawa, J., De Jesus, D.F., Kulkarni, R.N., 2017. Exploring inter-organ crosstalk to uncover mechanisms that regulate β-cell function and mass. European Journal of Clinical Nutrition 71, 896–903. https://doi.org/10.1038/ejcn.2017.13

Shyng, S.-L., Nichols, C.G., 1997. Octameric Stoichiometry of the KATP Channel Complex. J Gen Physiol 110, 655–664. https://doi.org/10.1085/jgp.110.6.655

Simopoulos, C., Christodoulou, E., Lambropoulou, M., Tsaroucha, A.K., Kakolyris, S., Polychronidis, A., Karayiannakis, A.J., Chatzaki, E., 2009. Neuropeptide urocortin 1 and its receptors are expressed in the human liver. Neuroendocrinology 89, 315–326. https://doi.org/10.1159/000187136

Simpson, S., Smith, L., Bowe, J., 2018. Placental peptides regulating islet adaptation to pregnancy: clinical potential in gestational diabetes mellitus. Current Opinion in Pharmacology 43, 59–65. https://doi.org/10.1016/j.coph.2018.08.004

Skow, M.A., Bergmann, N.C., Knop, F.K., 2016. Diabetes and obesity treatment based on dual incretin receptor activation: 'twincretins.' Diabetes, Obesity and Metabolism 18, 847–854. https://doi.org/10.1111/dom.12685

Slominski, A., PISARCHIK, A., TOBIN, D.J., MAZURKIEWICZ, J.E., WORTSMAN, J., 2004. Differential Expression of a Cutaneous Corticotropin-Releasing Hormone System. Endocrinology 145, 941–950. https://doi.org/10.1210/en.2003-0851

Smith, G.W., Aubry, J.M., Dellu, F., Contarino, A., Bilezikjian, L.M., Gold, L.H., Chen, R., Marchuk, Y., Hauser, C., Bentley, C.A., Sawchenko, P.E., Koob, G.F., Vale, W., Lee, K.F., 1998. Corticotropin releasing factor receptor 1-deficient mice display decreased anxiety, impaired stress response, and aberrant neuroendocrine development. Neuron 20, 1093–1102. https://doi.org/10.1016/s0896-6273(00)80491-2

Smith, L.I.F., Hill, T.G., Bowe, J.E., 2020. Generating Beta-Cell-Specific Transgenic Mice Using the Cre-Lox System. Methods Mol. Biol. 2128, 181–205. https://doi.org/10.1007/978-1-0716-0385-7_13

Smith, R., 2007. Parturition. New England Journal of Medicine 356, 271–283. https://doi.org/10.1056/NEJMra061360

Soares, M.J., 2004. The prolactin and growth hormone families: Pregnancy-specific hormones/cytokines at the maternal-fetal interface. Reproductive Biology and Endocrinology 2, 51. https://doi.org/10.1186/1477-7827-2-51

Sonagra, A.D., Biradar, S.M., K., D., Murthy D.S., J., 2014. Normal Pregnancy- A State of Insulin Resistance. J Clin Diagn Res 8, CC01–CC03. https://doi.org/10.7860/JCDR/2014/10068.5081

Song, Y., Manson, J.E., Tinker, L., Howard, B.V., Kuller, L.H., Nathan, L., Rifai, N., Liu, S., 2007. Insulin Sensitivity and Insulin Secretion Determined by Homeostasis Model Assessment (HOMA) and Risk of Diabetes in a Multiethnic Cohort of Women: The Women's Health Initiative Observational Study. Diabetes Care 30, 1747–1752. https://doi.org/10.2337/dc07-0358

Sorenson, R.L., Brelje, T.C., 2009. Prolactin Receptors Are Critical to the Adaptation of Islets to Pregnancy. Endocrinology 150, 1566–1569. https://doi.org/10.1210/en.2008-1710

Sorenson, R.L., Brelje, T.C., 1997. Adaptation of islets of Langerhans to pregnancy: beta-cell growth, enhanced insulin secretion and the role of lactogenic hormones. Horm. Metab. Res. 29, 301–307. https://doi.org/10.1055/s-2007-979040

Sorenson, R.L., Brelje, T.C., Roth, C., 1993. Effects of steroid and lactogenic hormones on islets of Langerhans: a new hypothesis for the role of pregnancy steroids in the adaptation of islets to pregnancy. Endocrinology 133, 2227–2234. https://doi.org/10.1210/endo.133.5.8404674

Spellacy, W.N., Goetz, F.C., Greenberg, B.Z., Ells, J., 1965. PLASMA INSULIN IN NORMAL MIDPREGNANCY. Am. J. Obstet. Gynecol. 92, 11–15. https://doi.org/10.1016/0002-9378(65)90099-2

Squillacioti, C., Pelagalli, A., Liguori, G., Mirabella, N., 2019. Urocortins in the mammalian endocrine system. Acta Vet Scand 61, 46. https://doi.org/10.1186/s13028-019-0480-2

Stalla, G.K., Bost, H., Stalla, J., Kaliebe, T., Dörr, H.G., Pfeiffer, D., von Werder, K., Müller, O.A., 1989. Human corticotropin-releasing hormone during pregnancy. Gynecol. Endocrinol. 3, 1–10. https://doi.org/10.3109/09513598909152447

Stancill, J.S., Osipovich, A.B., Cartailler, J.-P., Magnuson, M.A., 2018. Transgene-associated human growth hormone expression in pancreatic β-cells impairs identification of sex-based gene expression differences. American Journal of Physiology-Endocrinology and Metabolism 316, E196–E209. https://doi.org/10.1152/ajpendo.00229.2018

Steckler, T., Holsboer, F., 1999. Corticotropin-releasing hormone receptor subtypes and emotion. Biological Psychiatry 46, 1480–1508. https://doi.org/10.1016/S0006-3223(99)00170-5

Steiner, D.J., Kim, A., Miller, K., Hara, M., 2010. Pancreatic islet plasticity: Interspecies comparison of islet architecture and composition. Islets 2, 135–145. https://doi.org/10.4161/isl.2.3.11815

Stenbit, A.E., Tsao, T.-S., Li, J., Burcelin, R., Geenen, D.L., Factor, S.M., Houseknecht, K., Katz, E.B., Charron, M.J., 1997. GLUT4 heterozygous knockout mice develop muscle insulin resistance and diabetes. Nature Medicine 3, 1096–1101. https://doi.org/10.1038/nm1097-1096

Sun, X., Yu, W., Hu, C., 2014. Genetics of Type 2 Diabetes: Insights into the Pathogenesis and Its Clinical Application [WWW Document]. BioMed Research International. https://doi.org/10.1155/2014/926713

Swaroop, J.J., Rajarajeswari, D., Naidu, J.N., 2012. Association of TNF-α with insulin resistance in type 2 diabetes mellitus. Indian J Med Res 135, 127–130. https://doi.org/10.4103/0971-5916.93435

Taché, Y., Brunnhuber, S., 2008. From Hans Selye's Discovery of Biological Stress to the Identification of Corticotropin Releasing Factor signaling pathways: Implication in Stress-Related Functional Bowel Diseases. Ann N Y Acad Sci 1148, 29–41. https://doi.org/10.1196/annals.1410.007

Takefuji, M., Murohara, T., 2019. Corticotropin-Releasing Hormone Family and Their Receptors in the Cardiovascular System. Circulation Journal 83, 261–266. https://doi.org/10.1253/circj.CJ-18-0428

Tamás, G., Kerényi, Z., 2002. Current controversies in the mechanisms and treatment of gestational diabetes. Curr Diab Rep 2, 337–346. https://doi.org/10.1007/s11892-002-0024-3

Tasma, Z., Wills, P., Hay, D.L., Walker, C.S., 2020. Agonist bias and agonist-dependent antagonism at corticotrophin releasing factor receptors. Pharmacol Res Perspect 8. https://doi.org/10.1002/prp2.595

Tassava, T.M., Okuda, T., Romsos, D.R., 1992. Insulin Secretion From Ob/Ob Mouse Pancreatic Islets: Effects of Neurotransmitters [WWW Document]. The American journal of physiology. https://doi.org/10.1152/ajpendo.1992.262.3.E338

Thomson, M., 2013. The physiological roles of placental corticotropin releasing hormone in pregnancy and childbirth. J. Physiol. Biochem. 69, 559–573. https://doi.org/10.1007/s13105-012-0227-2

Thorens, B., 2014. Neural regulation of pancreatic islet cell mass and function. Diabetes, Obesity and Metabolism 16, 87–95. https://doi.org/10.1111/dom.12346

Toft-Nielsen, M.B., Damholt, M.B., Madsbad, S., Hilsted, L.M., Hughes, T.E., Michelsen, B.K., Holst, J.J., 2001. Determinants of the impaired secretion of glucagon-like peptide-1 in type 2 diabetic patients. J. Clin. Endocrinol. Metab. 86, 3717–3723. https://doi.org/10.1210/jcem.86.8.7750

Tomita, T., 2016. Insulin Resistance Syndrome in OB/OB Mouse [WWW Document]. URL https://austinpublishinggroup.com/endocrinology-diabetes/fulltext/ajed-v3-id1052.php (accessed 5.25.20).

Tsatsanis, C., Dermitzaki, E., Venihaki, M., Chatzaki, E., Minas, V., Gravanis, A., Margioris, A.N., 2007. The corticotropin-releasing factor (CRF) family of peptides as local modulators of adrenal function. Cell. Mol. Life Sci. 64, 1638–1655. https://doi.org/10.1007/s00018-007-6555-7

Tsuda, T., Takefuji, M., Wettschureck, N., Kotani, K., Morimoto, R., Okumura, T., Kaur, H., Eguchi, S., Sakaguchi, T., Ishihama, S., Kikuchi, R., Unno, K., Matsushita, K., Ishikawa, S., Offermanns, S., Murohara, T., 2017. Corticotropin releasing hormone receptor 2 exacerbates chronic cardiac dysfunction. J Exp Med 214, 1877–1888. https://doi.org/10.1084/jem.20161924

Turner, P.V., Brabb, T., Pekow, C., Vasbinder, M.A., 2011. Administration of Substances to Laboratory Animals: Routes of Administration and Factors to Consider. J Am Assoc Lab Anim Sci 50, 600–613.

Turok, D.K., Ratcliffe, S., Baxley, E.G., 2003. Management of Gestational Diabetes Mellitus. AFP 68, 1767–1772.

Vale, W., Spiess, J., Rivier, C., Rivier, J., 1981. Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. Science 213, 1394–1397. https://doi.org/10.1126/science.6267699

Van Assche, F.A., Aerts, L., Prins, F.D., 1978. A Morphological Study of the Endocrine Pancreas in Human Pregnancy. BJOG: An International Journal of Obstetrics & Gynaecology 85, 818–820. https://doi.org/10.1111/j.1471-0528.1978.tb15835.x

van de Bunt, M., Gloyn, A.L., 2012. A tale of two glucose transporters: how GLUT2 re-emerged as a contender for glucose transport into the human beta cell. Diabetologia 55, 2312–2315. https://doi.org/10.1007/s00125-012-2612-3

van der Meulen, T., Donaldson, C.J., Cáceres, E., Hunter, A.E., Cowing–Zitron, C., Pound, L.D., Adams, M.W., Zembrzycki, A., Grove, K.L., Huising, M.O., 2015. Urocortin3 mediates somatostatin-dependent negative feedback control of insulin secretion. Nat Med 21, 769–776. https://doi.org/10.1038/nm.3872

van Greevenbroek, M.M.J., Schalkwijk, C.G., Stehouwer, C.D.A., 2013. Obesity-associated low-grade inflammation in type 2 diabetes mellitus: causes and consequences. Neth J Med 71, 174–187.

Vandael, D., Gounko, N.V., 2019. Corticotropin releasing factor-binding protein (CRF-BP) as a potential new therapeutic target in Alzheimer's disease and stress disorders. Translational Psychiatry 9, 1–11. https://doi.org/10.1038/s41398-019-0581-8

Vanky, E., Zahlsen, K., Spigset, O., Carlsen, S.M., 2005. Placental passage of metformin in women with polycystic ovary syndrome. Fertil. Steril. 83, 1575–1578. https://doi.org/10.1016/j.fertnstert.2004.11.051

Vasavada, R.C., Garcia-Ocaña, A., Zawalich, W.S., Sorenson, R.L., Dann, P., Syed, M., Ogren, L., Talamantes, F., Stewart, A.F., 2000. Targeted expression of placental lactogen in the beta cells of transgenic mice results in beta cell proliferation, islet mass augmentation, and hypoglycemia. J. Biol. Chem. 275, 15399–15406.

Vaughan, J., Donaldson, C., Bittencourt, J., Perrin, M.H., Lewis, K., Sutton, S., Chan, R., Turnbull, A.V., Lovejoy, D., Rivier, C., 1995. Urocortin, a mammalian neuropeptide

related to fish urotensin I and to corticotropin-releasing factor. Nature 378, 287–292. https://doi.org/10.1038/378287a0
Vaughan, Joan, Donaldson, C., Bittencourt, J., Perrin, M.H., Lewis, K., Sutton, S., Chan, R., Turnbull, A.V., Lovejoy, D., Rivier, C., Rivier, J., Sawchenko, P.E., Vale, W., 1995. Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotropin-releasing factor. Nature 378, 287–292. https://doi.org/10.1038/378287a0
Voltolini, C., Battersby, S., Novembri, R., Torricelli, M., Severi, F.M., Petraglia, F., Norman, J.E., 2015. Urocortin 2 Role in Placental and Myometrial Inflammatory Mechanisms at Parturition. Endocrinology 156, 670–679. https://doi.org/10.1210/en.2014-1432
Voltolini, C., Battersby, S., Petraglia, F., Norman, J., 2012. Expression of CRH/urocortin gene family in mouse gestational tissues during late pregnancy. Presented at the 15th International & 14th European Congress of Endocrinology, BioScientifica.
Wadhwa, P.D., Garite, T.J., Porto, M., Glynn, L., Chicz-DeMet, A., Dunkel-Schetter, C., Sandman, C.A., 2004. Placental corticotropin-releasing hormone (CRH), spontaneous preterm birth, and fetal growth restriction: a prospective investigation. Am. J. Obstet. Gynecol. 191, 1063–1069. https://doi.org/10.1016/j.ajog.2004.06.070
Wadhwa, R.R., Cascella, M., 2020. Steady State Concentration, in: StatPearls. StatPearls Publishing, Treasure Island (FL).
Wallace, T.M., Levy, J.C., Matthews, D.R., 2004. Use and Abuse of HOMA Modeling. Diabetes Care 27, 1487–1495. https://doi.org/10.2337/diacare.27.6.1487
Wang, B., P., C.C., Pippin, J.J., 2014. Leptin- and Leptin Receptor-Deficient Rodent Models: Relevance for Human Type 2 Diabetes. Curr Diabetes Rev 10, 131–145. https://doi.org/10.2174/1573399810666140508121012
Wang, P., Fiaschi-Taesch, N.M., Vasavada, R.C., Scott, D.K., García-Ocaña, A., Stewart, A.F., 2015. Diabetes mellitus—advances and challenges in human β-cell proliferation. Nature Reviews Endocrinology 11, 201–212. https://doi.org/10.1038/nrendo.2015.9
Warren, W.B., Patrick, S.L., Goland, R.S., 1992. Elevated maternal plasma corticotropin-releasing hormone levels in pregnancies complicated by preterm labor. Am. J. Obstet. Gynecol. 166, 1198–1204; discussion 1204-1207. https://doi.org/10.1016/s0002-9378(11)90606-1
Watanabe, F., Oki, Y., Ozawa, M., Masuzawa, M., Iwabuchi, M., Yoshimi, T., Nishiguchi, T., Iino, K., Sasano, H., 1999. Urocortin in human placenta and maternal plasma. Peptides 20, 205–209. https://doi.org/10.1016/S0196-9781(98)00175-2
Webber, J., MacDonald, I.A., 1993. Metabolic actions of catecholamines in man. Baillière's Clinical Endocrinology and Metabolism, Catecholamines 7, 393–413. https://doi.org/10.1016/S0950-351X(05)80181-8
Weinhaus, A.J., Stout, L.E., Bhagroo, N.V., Brelje, T.C., Sorenson, R.L., 2007. Regulation of glucokinase in pancreatic islets by prolactin: a mechanism for increasing glucose-stimulated insulin secretion during pregnancy. J. Endocrinol. 193, 367–381. https://doi.org/10.1677/JOE-07-0043
Weyer, C., Hanson, R.L., Tataranni, P.A., Bogardus, C., Pratley, R.E., 2000. A High Fasting Plasma Insulin Concentration Predicts Type 2 Diabetes Independent of Insulin Resistance. Diabetes 49, 2094–2094.
Whitaker, R.C., Pepe, M.S., Seidel, K.D., Wright, J.A., Knopp, R.H., 1998. Gestational diabetes and the risk of offspring obesity. Pediatrics 101, E9. https://doi.org/10.1542/peds.101.2.e9
Wilcox, G., 2005. Insulin and Insulin Resistance. Clin Biochem Rev 26, 19–39.
Wiley, K.E., Davenport, A.P., 2004. CRF $_2$ receptors are highly expressed in the human cardiovascular system and their cognate ligands urocortins 2 and 3 are potent vasodilators: CRF $_2$ receptors in the human vasculature. British Journal of Pharmacology 143, 508–514. https://doi.org/10.1038/sj.bjp.0705985
Winkler, G., Cseh, K., Baranyi, E., Melczer, Z., Speer, G., Hajós, P., Salamon, F., Turi, Z., Kovács, M., Vargha, P., Karádi, I., 2002. Tumor necrosis factor system in insulin resistance in gestational diabetes. Diabetes Res. Clin. Pract. 56, 93–99. https://doi.org/10.1016/s0168-8227(01)00355-2

Winzell, M.S., Ahrén, B., 2007. G-protein-coupled receptors and islet function—Implications for treatment of type 2 diabetes. Pharmacology & Therapeutics 116, 437–448. https://doi.org/10.1016/j.pharmthera.2007.08.002

Wypior, G., Jeschke, U., Kurpisz, M., Szekeres-Bartho, J., 2011. Expression of CRH, CRH-related peptide and CRH receptor in the ovary and potential CRH signalling pathways. Journal of Reproductive Immunology 90, 67–73. https://doi.org/10.1016/j.jri.2011.04.009

Xiang, A.H., Peters, R.K., Trigo, E., Kjos, S.L., Lee, W.P., Buchanan, T.A., 1999. Multiple metabolic defects during late pregnancy in women at high risk for type 2 diabetes. Diabetes 48, 848–854. https://doi.org/10.2337/diabetes.48.4.848

Xin, Y., Kim, J., Okamoto, H., Ni, M., Wei, Y., Adler, C., Murphy, A.J., Yancopoulos, G.D., Lin, C., Gromada, J., 2016. RNA Sequencing of Single Human Islet Cells Reveals Type 2 Diabetes Genes. Cell Metabolism 24, 608–615. https://doi.org/10.1016/j.cmet.2016.08.018

Yamada, K., Yamakawa, K., Terada, Y., Kawaguchi, K., Sugaya, A., Sugiyama, T., Toyoda, N., 1999. Expression of GLUT4 glucose transporter protein in adipose tissue and skeletal muscle from streptozotocin-induced diabetic pregnant rats. Horm. Metab. Res. 31, 508–513. https://doi.org/10.1055/s-2007-978785

Yang, L.-Z., Tovote, P., Rayner, M., Kockskämper, J., Pieske, B., Spiess, J., 2010. Corticotropin-releasing factor receptors and urocortins, links between the brain and the heart. European Journal of Pharmacology 632, 1–6. https://doi.org/10.1016/j.ejphar.2010.01.027

You, X., Gao, L., Liu, J., Xu, C., Liu, C., Li, Y., Hui, N., Gu, H., Ni, X., 2012. CRH Activation of Different Signaling Pathways Results in Differential Calcium Signaling in Human Pregnant Myometrium before and during Labor. J Clin Endocrinol Metab 97, E1851–E1861. https://doi.org/10.1210/jc.2011-3383

You, X., Liu, J., Xu, C., Liu, W., Zhu, X., Li, Y., Sun, Q., Gu, H., Ni, X., 2014. Corticotropin-releasing hormone (CRH) promotes inflammation in human pregnant myometrium: the evidence of CRH initiating parturition? J. Clin. Endocrinol. Metab. 99, E199-208. https://doi.org/10.1210/jc.2013-3366

Young, A.A., Gedulin, B.R., Bhavsar, S., Bodkin, N., Jodka, C., Hansen, B., Denaro, M., 1999. Glucose-lowering and insulin-sensitizing actions of exendin-4: studies in obese diabetic (ob/ob, db/db) mice, diabetic fatty Zucker rats, and diabetic rhesus monkeys (Macaca mulatta). Diabetes 48, 1026–1034. https://doi.org/10.2337/diabetes.48.5.1026

Zaccardi, F., Webb, D.R., Yates, T., Davies, M.J., 2016. Pathophysiology of type 1 and type 2 diabetes mellitus: a 90-year perspective. Postgraduate Medical Journal 92, 63–69. https://doi.org/10.1136/postgradmedj-2015-133281

Zajdenverg, L., Negrato, C.A., Zajdenverg, L., Negrato, C.A., 2017. Gestational diabetes mellitus and type 2 diabetes: same disease in a different moment of life? Maybe not. Archives of Endocrinology and Metabolism 61, 208–210. https://doi.org/10.1590/2359-3997000000276

Zhang, J., Ma, S., Guo, C., Long, S., Wu, S., Tan, H., 2018. Research progress on etiology of gestational diabetes mellitus. Global Health Journal 2, 19–27. https://doi.org/10.1016/S2414-6447(19)30179-4

Zhang, Q., Ramracheya, R., Lahmann, C., Tarasov, A., Bengtsson, M., Braha, O., Braun, M., Brereton, M., Collins, S., Galvanovskis, J., Gonzalez, A., Groschner, L.N., Rorsman, N.J.G., Salehi, A., Travers, M.E., Walker, J.N., Gloyn, A.L., Gribble, F., Johnson, P.R.V., Reimann, F., Ashcroft, F.M., Rorsman, P., 2013. Role of KATP Channels in Glucose-Regulated Glucagon Secretion and Impaired Counterregulation in Type 2 Diabetes. Cell Metab 18, 871–882. https://doi.org/10.1016/j.cmet.2013.10.014

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., Friedman, J.M., 1994. Positional cloning of the mouse obese gene and its human homologue. Nature 372, 425–432. https://doi.org/10.1038/372425a0

Zhen-Juan, T., Ming-Jing, X., Chang-Hua, Q., Hong-Xia, Y., 2017. Long-term risk of metabolic disorders in gestational diabetes mellitus mothers and offspring. Biomedical Research 28.

Zhou, Y., Rui, L., 2013. Leptin signaling and leptin resistance. Front Med 7, 207–222. https://doi.org/10.1007/s11684-013-0263-5

Zhu, Y., Zhang, C., 2016. Prevalence of Gestational Diabetes and Risk of Progression to Type 2 Diabetes: a Global Perspective. Curr Diab Rep 16, 7. https://doi.org/10.1007/s11892-015-0699-x

Ẓmijewski, M.A., Slominski, A.T., 2010. Emerging role of alternative splicing of CRF1 receptor in CRF signaling. Acta Biochim Pol 57, 1–13.