Imperial College London

Biased Signalling of Dual GLP-1R/GCGR Agonists

A thesis submitted for the degree of Doctor of Philosophy at Imperial College London

Philip John Pickford

2020

Section of Endocrinology & Investigative Medicine
Division of Diabetes, Endocrinology & Metabolism
Department of Metabolism, Digestion and Reproduction
Imperial College London

Abstract

Dual agonists acting at both the glucagon-like peptide-1 receptor (GLP-1R) and glucagon receptor (GCGR) are a novel recent class of drugs which have been shown to simultaneously correct hyperglycaemia and cause sustained weight loss, making them a major drug class to treat the obese diabetic patient population. It is known that selectively activating certain intracellular pathways associated with a particular G protein-coupled receptor (GPCR), termed biased agonism, can simultaneously increase therapeutic efficacy and reduce associated side effects which can limit dosage of GPCR-targeting drugs. This approach has not been investigated in dual GLP-1R/GCGR agonists, yet it could improve the therapeutic efficacy of this drug class.

A GLP-1R/GCGR agonist peptide, “SRB103Gln3”, was discovered that displays significant reductions in β-arrestin recruitment at both receptors *versus* a comparator, “SRB103His3”, with both peptides maintaining full cAMP signalling. In hepatoma cells, SRB103Gln3 displayed more prolonged signalling than SRB103His3 after overnight stimulation, suggesting SRB103Gln3 could prolong signalling at the GCGR by reducing β-arrestin-mediated receptor desensitisation.

In lean and obese mice, SRB103Gln3 displayed greater anti-hyperglycaemic effects at prolonged timepoints compared to SRB103His3 with little reduction in acute food intake. Studies using *Gcgr*$^{-/-}$ mice were performed in an attempt to identify the contribution of GCGR to the observed effects. When administered chronically, SRB103Gln3 maintains its optimal anti-hyperglycaemic effects compared to SRB103His3, and both dual agonists displayed a trajectory suggesting greater weight loss compared to the GLP-1R mono-agonist liraglutide. In rats, SRB103Gln3 had profound effects on reducing food intake and weight loss compared to SRB103His3.

These findings highlight the benefit of selectively reducing β-arrestin recruitment associated with dual GLP-1R/GCGR agonist signalling, and could improve the therapeutic utility of this class of compounds.

Declaration of Contributors

The majority of the work presented in this thesis was performed by the author. All collaborations and assistance are described below:

Assistance with cAMP and β-arrestin assays was provided by Dr Ben Jones (Section of Investigative Medicine, Imperial College London)

Gcgr$^{fl/fl}$ mice were a kind gift from Dr Victoria Salem (Section of Investigative Medicine, Imperial College London)

Actb-*Cre-ERT2* and *Alb*-*Cre* mice were kindly provided by Prof David Carling (Faculty of Medicine, Imperial College London)

βArrβ$^{-/-}$ mice were kindly provided by Dr Alejandra Tomas and Dr Stavroula Bitsi (Section of Cell Biology and Functional Genomics, Imperial College London)

Radioimmunoassays were carried out by Mrs Zainab Mirza (Section of Investigative Medicine)

HPLC analysis of DPP-IV degradation was performed by Mr Zijian Feng (Section of Investigative Science, Imperial College London)

Assistance with *in vivo* experiments was provided by Dr Ben Jones, Mr Yateen Patel, Dr Maria Lucey, Miss Anne George and Miss Cecilia Dunsterville (all Section of Investigative Medicine, Imperial College London).

Copyright Declaration

Acknowledgements

I have enjoyed my PhD wholeheartedly, and a big part of that has been the fantastic people who have supported me along the way. I want to thank my primary supervisor Professor Sir Steve Bloom for allowing me the opportunity to undertake this research in his lab, along with his wonderful and witty advice. I would like to extend this to my secondary supervisor Dr James Minnion, who has always given me the opportunity to develop both my scientific and personal skills in drug discovery. And finally, a huge thank you to Dr Ben Jones who aided me day (and night) with unrelenting guidance on my project work, providing me with opportunities to advance my scientific career. Without their advice, both professional and personal, I wouldn't be where I am today.

Secondly, I want to say a huge thank you the wonderful people at Imperial, who have eased the pain of some occasionally stressful moments and provided many happy memories along the way. This includes Yat, Mariana, Anna, Maria, Aygul, Claire, Adrian, Katerina, Aaron, David, the G series team past and present and the many, many other great people who I haven't mentioned.

To my girlfriend Anne, thank you for looking after me during my writing up period and for making my life inside and outside of work better.

Finally, I want to save my greatest thanks to my family: Mum, Dad, Han and Will. Your constant belief, guidance, support, humour and love has made everything in my life so much better, and for that I will be forever grateful.

Table of Contents

Index of Figures

Index of Tables

Abbreviations

5-HT	serotonin
7TM	seven transmembrane
AAV	adeno-associated virus
AC	adenylyl cyclase
ACC	acetyl-coenzyme-A carboxylase
Actb	beta actin
ADP	adenosine diphosphate
AIB	2-aminoisobutyric acid
Alb	albumin
AMPK	adenosine monophosphate-activated protein kinase
AP2	adaptor protein-2
ATIIR	angiotensin II receptor
ATP	adenosine triphosphate
AUC	area under the curve
βArr	beta arrestin
BAT	brown adipose tissue
BBB	blood-brain barrier
BETP	4-(3-(benzyloxy)phenyl)-2-(ethylsulfinyl)-6-(trifluoro-methyl)pyramidine
BMI	body mass index
BRET	bioluminescence resonance energy transfer
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CHO	Chinese hamster ovary
CICR	calcium-induced calcium release
CNS	central nervous system
CREB	cAMP response element-binding protein
CRTC2	CREB-regulated transcription coactivator 2
SCF	cerebrospinal fluid
CVS	cardiovascular system
DAG	diacyl glycerol
DAPI	4’-6-diamidino-2-phenylindole
DEXA	dual-energy X-ray absorptiometry
DHG	desHis1-[Glu9]-glucagon(1-29)
DIO	diet-induced obese
DMEM	Dulbecco’s Modified Eagle Medium
DOR	delta opioid receptor
DPP-IV	dipeptidyl peptidase-4
ECL	extracellular loop
ELISA	enzyme-linked immunosorbent assay
Epac	exchange protein directly activated by cAMP
ERK1/2	extracellular signal-regulated kinase 1/2

Ex4	exendin-4
Ex9	exendin9-39
FASN	fatty acid synthase
FBS	foetal bovine serum
FDA	Food and Drug Administration
FFA	free fatty acid
FRET	Förster resonance energy transfer
FSK	forskolin
G6P	glucose-6-phosphate
G6Pase	glucose-6-phosphatase
G_{α}	G-protein alpha subunit
$G_{\beta/\gamma}$	G-protein beta/gamma subunits
GCGR	glucagon receptor
GDP	guanosine-diphosphate
GI	gastrointestinal
GIP	glucose-dependant insulinotropic polypeptide
GIPR	glucose-dependant insulinotropic polypeptide receptor
GK	Goto Kakizaki
Gln	glutamine
GLP-1	glucagon-like peptide-1
GLP-1R	glucagon-like peptide-1 receptor
GLUT4	glucose transporter-4
GP	glycogen phosphorylase
GPCR	G-protein coupled receptor
GRK	G protein receptor kinase
GSIS	glucose-stimulated insulin release
GTP	guanosine triphosphate
GTT	glucose tolerance test
GWAS	genome-wide association study
HEK	human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HFD	high-fat diet
His	histidine
HOMA	homeostatic model assessment
HOMA-β	homeostatic model assessment of beta cell function
HOMA-IR	homeostatic model assessment of insulin resistance
HTRF	homogenous time resolved fluorescence
HTS	high-throughput screen
IBMX	3-isobutyl-1-methylxanthine
ICL	intracellular loop
IgG4	immunoglobulin G-4
IGGTT	intragastric glucose tolerance test
IKKβ	inhibitor of nuclear factor kappa beta
IL	interleukins

i.p.	intraperitoneal
IP-1	intervening peptide-1
IPGTT	intraperitoneal glucose tolerance test
IR	insulin receptor
IRS-1	insulin receptor substrate-1
ITT	insulin tolerance test
i.v.	intravenous
IVGTT	intravenous glucose tolerance test
JAK-STAT	Janus kinase-signal transducer and activator of transcription
JNK	Janus kinase
KATP	ATP-activated protein channel
KCNJ11	potassium inwardly-rectifying channel subfamily J member 11
KOR	kappa opioid receptor
LgBit	large-subunit
LKB-1	liver kinase B1
M2R	M2 muscarinic receptor
MCP-1	monocyte chemotactic protein-1
MOR	mu opioid receptor
MRI	magnetic resonance imaging
NAFLD	non-alcoholic fatty liver disease
NEFA	non-esterified fatty acid
NEP-24.11	neutral endopeptidase 24.11
NF-κB	nuclear factor kappa-light-chain enhancer of activated beta cells
NIPHS	non-insulinoma pancreatogenic hypoglycaemia syndrome
NMR	nuclear magnetic resonance
NNT	nicotinamide nucleotide transhydogenase
NZO	New Zealand Obese
OGTT	oral glucose tolerance test
OLETF	Otsuka Long-Evans Tokushima Fat
OXM	oxyntomodulin
PC	protein convertase
PCA	protein complementation assay
PCR	polymerase chain reaction
PEG	polyethylene glycol
PEPCK	phosphoenolpyruvate carboxykinase
Phe	phenylalanine
PI3K	phosphatidylinositol 3’-kinase
PK	pharmacokinetics
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PPARγ	peroxisome proliferator-activated receptor gamma
Ppg	preproglucagon
PTX	pertussis toxin

PYY	peptide YY
qPCR	quantitative polymerase chain reaction
RAMP2	receptor activity modifying protein 2
RBP4	retinol binding protein 4
RIA	radioimmune assay
RPMI	Rosa Park Memorial Institute
ROS	reactive oxygen species
RYGB	Roux-en-Y gastric bypass
s.c.	subcutaneous
SEM	standard error of the mean
Ser	serine
SFM	serum-free media
SGLT2	sodium-glucose co-transporter 2
shRNA	short hairpin ribonucleic acid
siRNA	small interfering ribonucleic acid
SLC30A8	solute carrier family 30 member 8
SmBit	small-subunit
T2DM	type 2 diabetes mellitus
TALEN	transcription activation-like effector nuclease
TCF7L2	transcription factor 7-like 2
TM	transmembrane
TNF-α	tissue necrosis factor alpha
TORC2	transducer of regulated CREB protein-2
TR-FRET	time-resolved Förster resonance energy transfer
Tyr	tyrosine
TZD	thiazolidinedione
V2R	V2 vasopressin receptor
VSG	vertical sleeve gastrectomy
WAT	white adipose tissue
WFI	water for injection
WHO	World Health Organisation
WT	wild type
ZDF	Zucker Diabetic Fatty
ZF	Zucker Fatty

1 Introduction

1.1 Type 2 diabetes mellitus

Diabetes mellitus, usually shortened to diabetes, is a metabolic disease caused by chronic hyperglycaemia, stemming from the body's inability to adequately control fluctuations in circulating glucose. The clinical definition of diabetes is fasting blood glucose of ≥7mm/kg, and glycated haemoglobin (HbA1c) of ≥6.5%. This is the result of two main facets. Type 1 is an autoimmune disease, where the body's immune system attacks and destroys the insulin-producing β cells of the pancreas. The second form, type 2, results from reduced insulin sensitivity, meaning that the endogenous insulin is not able to reduce circulating glucose levels and consequently leading to β cell death. It is estimated that there were 463 million people worldwide with diabetes in 2019, of which 90% of these had type 2 diabetes mellitus (T2DM)[5]. Treating type 2 typically involves boosting endogenous insulin production and secretion and improving the body's ability to utilise insulin.

T2DM is one of the top ten causes of early mortality[6], but is considered to be one of the most preventable. By 2045, projections forecast around 600-700 million people will have T2DM[7] and in 2019, T2DM was attributed to an estimated 2.2 million deaths, affecting both developed and developing countires[7,8]. Secondary to the mortality associated with T2DM, is the vast cost associated with treating T2DM. The global health expenditure to treat diabetes could rise steeply to $2.5 trillion by 2030[9], and this is expected to grow exponentially unless safe, efficacious and affordable therapies to treat T2DM are developed.

1.1.1 Physiological glucose control

Glucose is tightly regulated in healthy humans despite large fluctuations in glucose requirements and circulatory loads (i.e. during exercise and after meals). This process involves complex coordination between multiple organs including the pancreas, brain, liver, adipose tissue, kidney and intestine. The pancreas, specifically the islets of Langerhans, is the key tissue involved in glucose homeostasis. The islets are highly vascularised, allowing for rapid detection of fluctuating blood glucose concentrations[10]. Insulin, secreted from the β cells in the islet, is responsible for reducing circulating glucose levels. Insulin reduces glycaemia by increasing transcription rates of glucose

transporter-4 (GLUT4) to increase intracellular glucose flux, increasing expression of enzymes responsible for glycogenesis (the formation of glycogen from glucose). Insulin also reduces expression of enzymes responsible for glycogenolysis (the breakdown of glycogen to glucose) and gluconeogenesis (*de novo* synthesis of glucose from non-carbohydrate sources such as amino acids)[11]. Conversely glucagon, which is secreted by the α cell in the islet in response to hypoglycaemia, increases expression of enzymes involved in both gluconeogenesis and glycogenolysis, as well as inhibition of glycolysis (the metabolic breakdown of glucose), thereby increasing glycaemia through accelerated hepatic glucose output[2].

The theory of bi-hormonal glucose homeostasis, whereby glucagon and insulin work in opposing manners to maintain euglycaemia was first proposed in 1971 by Roger Unger and remains a reasonable description of glucose regulation. Interestingly, the receptors of both hormones, the insulin receptor (IR) and glucagon receptor (GCGR), are present on both the α cell and β cell. This suggests feedback mechanisms between the two hormones allow for paracrine control of secretion of each hormone[12,13].

Insulin secretion occurs through detection of high glucose levels, in a process called glucose-stimulated insulin secretion (GSIS) (Figure 1.1). However the model of GSIS is too simplistic, as the pancreatic β cell secretes insulin in response to parasympathetic innervation[14], amino acid sensing[15] and insulinotropic hormones secreted post-prandially[16]. These post-prandial hormones, called incretins, are secreted from the gastro-intestinal (GI) tract. An important function of the incretins is to relay information between the gut and pancreas, which was demonstrated when oral glucose loads resulted in significantly greater insulin secretion compared to the equivalent intravenous (i.v.) dose[17]. This so-called “incretin effect” is predicted to account for approximately 70% of post-prandial insulin secretion[17,18], emphasising the importance of factors outside of GSIS in provoking insulin secretion. The two prominent incretins involved in the “incretin effect” are glucagon-like peptide-1 (GLP-1) and glucose-dependant insulinotropic peptide (GIP). These two incretins are secreted from the intestinal L- and K-cells and activate their cognate receptors the GLP-1 receptor (GLP-1R) and GIP receptor (GIPR), which are present on the β cell[19]. The relative importance between GLP-1R or GIPR stimulation to augment insulin

release physiologically has now been better defined. The development of transgenic mice and antagonists to the receptors has led to the conclusion that GLP-1 reduces gastric emptying rates and is a more potent inhibitor of glucagon secretion[20], whilst GIP is the more powerful post-prandial insulinotropic incretin[21-23]. Interestingly, in cases of T2DM, GLP-1 function is still maintained, whereas that of GIP is completely lost[20,21]. Similarly, glucose is a key stimulant of GLP-1 secretion whereas dietary fat and protein are the more potent stimulators of GIP secretion, further suggesting divergent physiological roles[24-27].

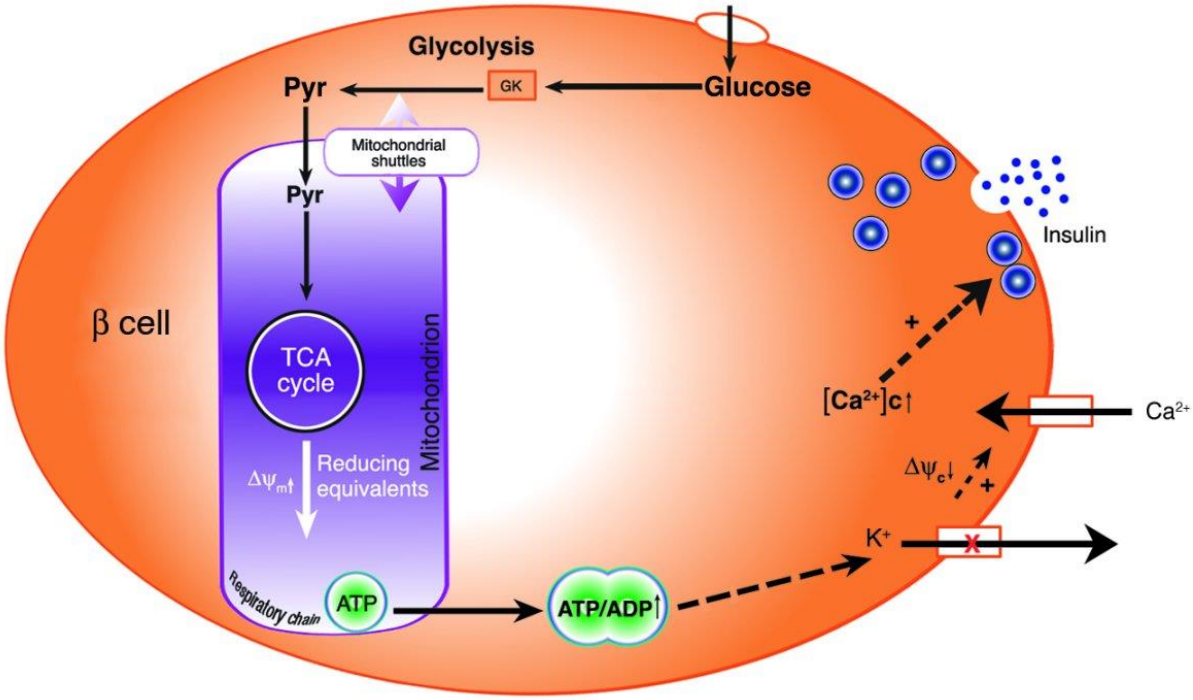

Figure 1.1-Schematic of glucose-stimulated insulin secretion from the pancreatic beta cell

Glucose enters the β cell through GLUT4 and is converted to pyruvate (Pyr) by glucokinase (GK). Pyruvate enters the mitochondrial tricarboxylic acid (TCA) cycle to produce adenosine triphosphate (ATP). Elevated ATP levels result in closure of the K_{ATP} channels, depolarising the membrane which opens Ca_V channels. This subsequent influx of Ca2+triggers insulin secretion from the β cell. Figure adapted from Brownlee, 2003[4].

1.1.2 Pathophysiology of type 2 diabetes mellitus

As mentioned, T2DM is associated with chronic hyperglycaemia, resulting from a failure of insulin to signal to insulin sensitive tissue to store circulating glucose. In understanding type 2 diabetes, it is important to discuss the two main facets of its pathophysiology: Impaired insulin sensitivity and impaired insulin secretion.

1.1.2.1 Impaired insulin sensitivity

Firstly, T2DM is typically associated with a reduction in insulin sensitivity. Insulin resistance is defined as the inability for insulin to signal through its receptor, resulting in impaired insulin-mediated glucose uptake into insulin-sensitive cells (e.g. adipose tissue and skeletal muscle). There is also impaired inhibition of glucagon secretion post-prandially, which has been suggested to contribute to hyperglycaemia through excessive hepatic glucose production. This results in prolonged hyperglycaemia, of which the consequences have been briefly discussed.

The pathophysiology of insulin resistance is not completely known; however, a number of theories and mechanisms have been hypothesised. There is a close association between increased adiposity and an increasing prevalence of insulin resistance[28], which is discussed in in section 1.2. Obese individuals can present with high circulating concentrations of free fatty acids (FFAs), which can be toxic to the β cell in chronic doses, induce an excessive inflammatory response[29,30] and increase oxidative stress in insulin-sensitive tissues[31], resulting in insulin resistance. Conversely, FFAs are a stimulator of insulin secretion (FFA receptors are functionally expressed on the β cell), therefore elevated circulating FFAs plays both positive and negative roles in T2DM pathogenesis[32]. Indeed, obese rodents treated with antioxidants display improved insulin sensitivity[33] and reduction of plasma fatty acids is extensively linked in multiple species to improved insulin sensitivity[34-36], thus linking obesity, oxidative stress and insulin resistance.

Inflammatory mediators, such as tissue necrosis factor α (TNF-α), monocyte chemotactic protein-1 (MCP-1) and interleukins (IL) are upregulated in cases of insulin resistance[37,38]. These factors can directly affect IR signalling by increasing phosphorylation rates of insulin receptor substrate-1 (IRS-1)[39]. IRS-1 acts as an adaptor protein in insulin-sensitive cells, transducing the signal from the activated IR to other intracellular pathways. Therefore, if IRS-1 function is altered, the ability of insulin to signal is compromised. Interleukins such as IL-6 have the capacity to degrade IRS-1[40] and activate key inflammatory pathways, such as the inhibitor of nuclear factor kappa B/nuclear factor kappa-light-chain-enhancer of activated B cells (IKKβ/NF-κB) and Janus N-terminal kinase (JNK) pathways impair physiological IRS-

1 phosphorylation[41]. This provides another mechanism linking inflammation to insulin resistance. TNF-α has also been shown to reduce GLUT4 expression, thus reducing the influx of glucose into insulin-sensitive cells[42].

Mitochondrial dysfunction from nutrient overload can cause oxidative stress[43,44] resulting in an overload of free radicals in insulin-sensitive tissues such as adipose tissue and skeletal muscle. As glucose and free fatty acid levels rise in pre-diabetes from over-consumption, the production of reactive oxygen species (ROS) increases in the mitochondrial TCA cycle beyond the capabilities of intracellular antioxidant control[45-47]. Indeed, while ROS are important signalling molecules in IR signalling[48,49], excessive ROS production leads to cellular oxidative stress which culminates in an inability to respond to insulin. Diabetic mice display an increase in biomarkers for oxidative stress in the adipose tissue, and treatment of wild-type adipocytes *ex vivo* with glycated albumin (which is enhanced in diabetes) resulted in the production of free radicals[50]. Conversely, insulin resistant mice treated with antioxidants display an improvement in insulin sensitivity further implicating oxidative stress as a key regulator in insulin resistance[33]. This implies that supra-physiological levels of ROS production do indeed contribute to insulin resistance, and could be an interesting proposition as a therapeutic target for pre-diabetic insulin resistance.

1.1.2.2 Impaired insulin secretion

T2DM is also manifested by a loss of β cell mass and function alongside the lack of insulin sensitivity. Indeed, T2DM patients present with increased β cell apoptosis as well as reduced β cell mass[51-53]. Whilst obesity is associated with T2DM, most obese individuals are able to secrete sufficient insulin levels to nullify excessive glucose loads, hence not all obese people are diabetic. Studies evaluating β cell mass show that obese, non-diabetic humans have a relative increase in β cell mass (to compensate for excessive circulating nutrient loads), however obese and non-obese diabetic patients display a 40-60% reduction in β cell mass[51,52]. This suggests that β cell mass is reduced as hyperglycaemia progresses towards T2DM.

One of the most discussed mechanisms of β cell failure in T2DM is glucolipotoxicity. Here, chronic exposure to high circulating FFA (lipotoxicity) and glucose (glucotoxicity)

work synergistically to cause β cell death, resulting in a vicious circle whereby high circulating nutrient levels impair insulin secretion, which increases nutrient circulating levels and so on[54,55]. Whilst acute exposure to FFA produces a small insulin secretory response[32,56], chronic exposure conversely inhibits secretion[57,58]. Links between FFA levels and β cell death is debated, as it is difficult to confirm the FFA levels at the β cell interface and FFA infusion studies can use supraphysiological FFA concentrations[59]. However, studies have shown that FFAs inhibit insulin gene expression *ex vivo*, which precedes abnormalities in secretion patterns in rats[60]. Glucotoxicity is well regarded for causing β cell mass loss, where in rodent and human with T2DM, studies have shown close temporal correlations between loss of GSIS and increasing glycaemia[61-64]. Mechanisms underlying how glucolipotoxicity induces β cell dysfunction and death include oxidative stress from excessive ROS generation in the β cell[65], disruption of physiological gene transcription[66] and increased rates of apoptosis[67,68].

Chronic exposure to hyperglycaemia itself also alters β cell development and differentiation rates, associated with gene expression changes termed "dedifferentiation", affecting physiological β cell metabolic pathways[69-71]. Interestingly, in studies investigating dedifferentiation in rats, FFA levels remained the same whilst hyperglycaemia ensued, insinuating dedifferentiation was attributable only to hyperglycaemia and not to any lipotoxic effects[69].

Genome wide association studies (GWAS) have provided a powerful tool in highlighting genetic mutations which can pertain towards the development of T2DM. Some of these mutations are associated with genes involved in insulin secretion, such as *KCNJ11* (potassium inwardly-rectifying channel subfamily J member 11)[72,73], *TCF7L2* (transcription factor 7-like 2)[74,75] and SLC30A8 (solute carrier family 30 member 8)[74,76] amongst others[77]. These GWAS findings further suggest that impairments in insulin secretion do occur before the disease sets in, and β cell defects are present prior to the onset of T2DM, suggesting impaired insulin secretion is part of T2DM pathogenesis.

1.2 "Diabesity"

Obesity is a global healthcare crisis. The latest available World Health Organisation (WHO) statistics estimate that 650 million adults worldwide are obese (Body mass index (BMI) ≥ 30kg/m^2), whilst another 1.3 billion are clinically overweight (BMI ≥ 25kg/m^2)[78]. The estimated number of people with obesity has tripled since 1975 and if current trends continue, by 2030, 58% of adults globally will be obese or overweight[79].

The aetiology of obesity is quite simple to describe. It is the consequence of surplus caloric intake *versus* caloric expenditure culminating in excessive adipose tissue accumulation. The accepted reason for rapid increases in obesity rates are linked to the modern lifestyle, which includes an increase in readily available energy rich fast-food, increased use of public transport, sedentary work forms and urbanisation tipping the balance toward excessive energy intake[80]. As globalisation expands the horizon of cheap, fast food and increased automation to developing countries, obesity is considered a health crisis in both developed and developing countries[81-83]. The economic cost of treating obesity are vast, costing the NHS at least £6.1 billion in 2015, and is estimated to rise to nearly £10 billion by 2050[84]. Since 1975, WHO have emphasised that the cost of managing obesity and its related disorders has sharply risen. It is therefore of vital importance that obesity is successfully combated to prevent a future medical crisis and economic ruin of healthcare systems.

1.2.1 Obesity and its associated diseases

Obesity is linked to the onset of numerous fatal comorbidities. According to WHO, cardiovascular disease, including myocardial infarction and stroke accounted for 31% of global deaths in 2016, is greatly associated with an unhealthy lifestyle including diet and obesity[85]. Obesity has also been linked to several forms of cancer, which is the second leading cause of global fatalities. A pooled meta-analyses of European cancer cases suggests approximately one in three patients with cancer were obese, with a strong linearity between BMI increase and risk of developing cancer[86]. It is also acknowledged that losing weight is associated with a decreased diagnosis of cancer[87,88]. The mechanisms leading to both of these diseases are not fully

understood, but it is known that obesity can result in an increase in circulating inflammatory markers, increased circulating lipids and alterations in circulating sex hormone concentrations[89,90]. The culmination of these events is detrimental to the physiology of most organs; therefore, the consequences of obesity clearly extends beyond an expanding waistline.

1.2.2 Obesity and type 2 diabetes mellitus

Obesity is most readily associated with the onset of T2DM with 90% of adults suffering from T2DM estimated to be overweight or obese[8]. This strong relationship between obesity and diabetes has resulted in the phrase “diabesity” to be developed, describing the obese diabetic patient population. However, not all obese individuals develop T2DM suggesting that the pathophysiological links extend beyond increased adiposity. A number of hypotheses exist linking obesity to T2DM.

The “lipid overflow hypothesis” postulates that the oversaturation of circulating lipids for storage results in lipids being deposited in insulin-sensitive tissues that do not normally accommodate large lipid stores[91]. Here, increasing lipid concentrations in non-adipose tissue results in increased lipotoxicity due to dysfunctional lipid oxidation, which has been shown to interact with and disturb physiological insulin signalling in these tissues[92]. This model is supported by rodent models of lipodystrophy which are lean, but suffer from the same metabolic profiles of obesity-induced diabetes[93,94]. Not only can the lipotoxicity affect insulin-sensitive tissues, but also the insulin-producing pancreatic β cell. Certain rodent models of obesity, such as the New Zealand Obese (NZO) mouse, develop insulin resistance leading to type 2 diabetes, characterised by hypertrophy and eventual death of the β cells. Interestingly, NZO mice fed on a low carbohydrate, high fat diet develop obesity and insulin resistance but not β cell failure[95], whereas NZO mice fed high fat, high carbohydrate diets quickly develop β cell failure, suggesting oversaturation of both carbohydrates and fat is required for β cell failure with the lipid overflow hypothesis[96]. Interestingly, T2DM patients who follow a low-carbohydrate diet show better improvements in glucose control, compared to isocaloric standard diets or low-fat isocaloric diets[97,98]. However, compliance and long-term safety with drastic diet change is still unknown and therefore requires the guidance of healthcare professionals

Secondly, the “adipokine hypothesis” suggests adipocytes acting as a secretory tissue for many endocrine and inflammatory markers (called adipokines) can themselves be detrimental to insulin-sensitive tissues. The expansion of the adipose tissue in obesity increases the circulating levels of adipokines which causes further damage to insulin-sensitive and -producing tissues. Adipokines which improve insulin secretion and sensitivity, such as adiponectin and adipolin, are reduced in obese individuals and contributes to the worsening of insulin sensitivity resulting in T2DM[99,100]. Other adipokines such as retinol binding protein 4 (RBP4), an adipokine which inhibits insulin signalling in skeletal muscles, and resistin and visfatin, which have proinflammatory properties, are upregulated adipokines in obese individuals, progressing insulin resistance[101]. Leptin, an adipocyte-derived factor which induces satiety, is an adipokine which regulates energy homeostasis by decreasing food intake and increasing energy expenditure[102]. Physiologically, it is prevalent at levels proportional to adipose tissue mass, suggesting an important role in regulating adipose tissue mass[103]. Leptin has also been shown to have direct and indirect actions on glucose homeostasis and insulin sensitivity through activation of the Janus kinase-signal transducer and activator of transcription (JAK-STAT), extracellular signal-regulated kinase 1/2 (ERK1/2) and adenosine monophosphate-activated protein kinase (AMPK) pathways[104-106]. In obese individuals, leptin is secreted from adipose tissue at vast quantities, and exogenous administration of leptin does not improve symptoms in most cases of obesity, suggesting leptin resistance is prevalent in most obese individuals[107,108]. Non-esterified fatty acids (NEFAs), also secreted by adipocytes, play a role in physiological insulin secretion[109,110], however when secreted in great quantities in obese people, NEFA overexposure has been linked to both progression of insulin resistance and β cell loss[111].

The “inflammation hypothesis” is linked to the adipokine hypothesis. This postulates that chronic inflammation produced by the altered secretion of overloaded adipocytes leads to damaging of insulin-sensitive tissues and insulin-secreting β cell. Excessive adipose accumulation leads to an increased circulating level of macrophages derived from adipocytes[112,113], and the levels of adipose tissue macrophages correlates with adipose insulin resistance[114,115]. These macrophages act as “cytokine reservoirs” for cytokines such as interleukin-1 (IL-1), IL-6 and TNF-α amongst others, which have been shown to affect β cell function, glucose disposal and adipose capabilities which

ultimate reduces insulin sensitivity[116-118]. Therefore, as the level of adiposity increases, so does the level of activated macrophages which ultimately leads to degeneration of physiological glucose management.

1.3 Treatment options for T2DM

1.3.1 Pharmacological

The link between obesity and risk of T2DM is very strong. Increasing BMI is associated with a higher risk of developing T2DM[119-122], while it is estimated that approximately 50-60% of T2DM cases could have been prevented if the BMI was in a normal range (<25kg/m2)[123-127]. Alongside the fact that 90% of T2DM patients are obese or overweight[128], it is logical that treatments for T2DM should also aim to tackle weight loss. Weight loss management through diet and exercise have been shown to significantly improve insulin sensitivity[129-132] and reduce T2DM diagnoses[133-135], even if the weight loss is moderate[136]. However, these prospective studies have not considered that weight loss due to lifestyle changes in obese individuals is usually transient due to the myriad of metabolic and physiological adaptations[137-139]. Therefore, therapeutic intervention is, in most cases, the only option to induce sustained weight loss and improve T2DM.

1.3.1.1 Insulin analogue therapy

T2DM is manifested with an inability to secrete insulin to counteract the raised glycaemia. To overcome this, exogenous insulin can be administered, with recombinant or synthetic analogues of insulin replacing purified porcine insulin. Insulin analogues are either fast-acting such as lispro, aspart or glulisine or long-acting such as detemir, degludec and glargine. Fast acting analogues are injected directly after a meal to provide protection agonists post-prandial hyperglycaemia while long acting analogues provide steady, day-long baseline levels of insulin to maintain euglycaemia following a once-daily injection[140].

Short-term insulin therapy may work best to preserve β cell integrity, as shown in recent human studies[141,142]. In newly diagnosed T2DM individuals, long-term insulin

therapy results in improved β cell function and long-term glycaemic control, more so than diet and lifestyle changes alone[143-145]. However, insulin is synonymous with weight gain due to its orexigenic properties and its anabolic effects at adipose tissues[146]. Weight gain is particularly undesirable in T2DM patients with the close association with obesity. Insulin therapy also presents a large risk of hypoglycaemia, when the insulin level outweighs that of carbohydrate intake. Finally, insulin glargine has been linked to an increased risk of cancer, hypothetically due to its affinity to the insulin-like growth factor I receptor (IGF-1R)[147]. Therefore, insulin therapy cannot be considered the "silver bullet" of T2DM treatments alone[148,149].

1.3.1.2 Metformin

Metformin is on the WHOs Essential Medicines list and is one of the most prescribed drugs globally as a first line treatment for T2DM. It primarily acts by reducing hepatic glucose output by activating liver kinase B1 (LKB-1) which, through regulation of AMPK and transducer of regulated CREB protein 2 (TORC2), reduces transcription of gluconeogenic enzymes[150]. This mechanism of action also increases peripheral glucose uptake, thus metformin improves insulin sensitivity[151]. In addition to this, metformin displays beneficial cardiovascular and metabolic effects, making it the first line treatment for T2DM.

In longitudinal studies, metformin treatment alone was shown to reduce fasting glucose levels and HbA1c levels in T2DM patients who are both standard weight BMI ≤ 25kg/m$_2$)[152-154], overweight (BMI 25 – 30kg/m$_2$)[155-158] and obese (BMI > 30kg/m$_2$)[152,159-161]. However, the efficacy of metformin can reduce after prolonged treatment, therefore it can be combined with a second anti-diabetes medication (such as the ones listed below). Metformin has been shown to improve T2DM symptoms as a combination therapy with insulin therapy[162], sulphonylureas[155,163-165], SGLT2 inhibitors[166-168], meglitanides[157,169,170] and thiazolidinediones[171] compared to either therapy alone, with the same or fewer recorded side effects. Metformin has also been shown to produce a small weight loss over extended treatment periods[172-175], however this is likely a secondary affect to decreased appetite and side effects. A further benefit of metformin is it also improves fatty liver index scores by reducing circulating lipid levels and improving fatty liver[176].

1.3.1.3 Other diabetes pharmacotherapies

Unlike metformin which reduces hepatic glucose output, sulphonylureas and meglitinides act on the β cell K_{ATP} channel to increase the resting membrane potential to augment insulin secretion[177,178]. Therefore, both of these therapies act to increase insulin secretion and are used as an adjuvant to T2DM treatment. Whilst both of these drug classes are sufficient at correcting the hyperglycaemia associated with T2DM, treatment with either can result in weight gain making them undesirable for treatment of the 90% of T2DM patients who are obese[179,180]. A further complication for the classes of drugs is, enhanced insulin secretion can result in life-threatening hypoglycaemia[181]. This is less of a problem with meglitinides as they have a shorter duration of action[182]. Sulphonylureas have also been linked to a low but noticeable rate of cardiovascular problems[183]. For this reason, they are considered as a "second-line" treatment to use when insulin treatment fails, and can be used in combination with other drugs such as metformin[184].

Thiazolidinediones (TZDs) are another class of drugs used to improve insulin sensitivity. TZDs activate peroxisome proliferator-activated receptor γ (PPARγ), a nuclear receptor which alters transcription of genes in adipose, skeletal muscle and the liver associated with glucose and lipid metabolism and homeostasis[185]. TZD show promise in the clinic to improve glycaemic control, and have been shown to be as good or better than metformin[162,186-189] and sulphonylureas[164,190-192]. However, due to the activation of a non-diabetes-specific pathway by TZDs, they are associated with serious potential side effects including increased cardiac risk, oedema and increased bone fracturing[193-195]. Indeed, only two TZDs are used in clinic today due to the excessive side effects; pioglitazone and rosiglitazone. In addition to the poor side effect profile, TZDs cause weight gain[196], which makes them unsuitable for many T2DM patients who are obese.

A more recent advance in T2DM therapy is the development of the SGLT2 inhibitors. SGLT2 is found in the renal nephrons, and causes reabsorption of glucose back into the circulation from the renal lumen[197]. SGLT2 inhibitors, also known as gliflozins, inhibit the reabsorption which culminates in the glucose being excreted in the urine. Unlike sulphonylureas and meglitinides, SGLT2 inhibitors do not stimulate insulin

secretion, therefore there is a considerably lower risk of hyperglycaemia[198]. As well as inhibiting glucose reabsorption, they also inhibit sodium reabsorption, causing a reduction in blood pressure[199,200]. As these are not insulin secretagogues, there is no weight gain observed in SGLT2 inhibitor therapy, small weight loss of 2-4kg observed in longitudinal human studies[201,202]. Combination of SGLT2 inhibitors and metformin are therefore and interesting combination therapy, and does show clinical promise, however it is associated with an increased risk of diabetic ketoacidosis[203].

In summary, there are exciting and efficacious pharmacotherapies available which can improve glycaemic control associated with T2DM. However, these therapies are only sufficient at treating the result of T2DM and have minimal effects on reversing the cause of T2DM. In addition, they are generally poor when administered as a monotherapy and associated with side effects when administered in combination. Additionally, they are at best poor at inducing weight loss, and in some cases facilitate weight gain. Therefore, there is still a need for efficacious treatments which correct glycaemic control in conjunction with causing meaningful and sustained weight loss.

1.3.2 Bariatric surgery

The efficacy of weight loss alone on T2DM remission is remarkable[133-135], however the prevalence of a long, sustained weight loss by means of lifestyle alterations is low in obese individuals. Bariatric surgery is widely regarded as the most successful treatment for reducing obesity and improving concomitant T2DM. Here, the stomach pouch is reduced in size, which can be performed alongside intestinal bypass. It leads to a substantial and sustained weight loss alongside a near total remission of diabetes[204,205]. Despite its usefulness in remitting obesity and T2DM, bariatric surgery comes with noticeable mortality risks due to its invasive nature[206], requires extensive effort in pre- and post-operative care on behalf of the patient and healthcare worker, they are an expensive therapeutic option. Therefore, surgery is usually only designated for morbidly obese individuals, or obese individuals with underlying obesity-related illnesses[207].

1.3.2.1 Potential mechanisms leading to T2DM remission

Remission in diabetes in bypass patients is quick, preceding that of extensive weight loss[208-211]. Therefore, in the case of bariatric surgery, some of the mechanisms which improve anti-hyperglycaemia are believed to be separate from that of just losing weight[212]. There have been a number of theories which have been suggested to explain the success of bariatric surgery. These include malabsorption of macronutrients such as fats and carbohydrates which decreases stress on the β cell[213], reduced "anti-incretin" secretion from the proximal intestine after bypass resulting in increased incretin secretion[214], increased bile acid and fibroblast growth factor-19 (FGF-19) levels which improve glucose and lipid homeostasis[215,216] and changes in the microbiome leading to improve glucose homeostasis[217].

However, the best regarded explanation is the change in incretin levels witnessed post-bariatric surgery. Incretins are secreted post-prandially from the gut, producing a myriad of peripheral and centrally-mediated effect which produce satiety, increased insulin secretion and improved insulin tolerance[218-221]. The incretin effect has also been suggested to promote weight loss through satiety and increased energy expenditure[218,222], therefore it provides a multifaceted approach to combatting diabetes. They provide a link between the gut and the pancreas, allowing for rapid and efficient processing of excess glucose from the meal into tissue which can store the excess glucose. Key incretins which are observed as being elevated immediately after bariatric surgery are GLP-1, GIP, peptide YY (PYY) and oxyntomodulin (OXM). T2DM patients regularly present with an ablated incretin response post-prandially, which may contribute to the diabetic phenotype[223-225], suggesting that utilising and restoring the incretin response in T2DM patients is a robust method of treating T2DM. Intestinal bypass surgery techniques, such as Roux-en-Y gastric bypass (RYGB) are associated with improved diabetic outcomes compared to other gastro-restrictive procedures such as vertical sleeve gastrectomy (VSG), and RYGB results in greater secretion of GLP-1 and PYY[226]. Co-infusion of GLP-1, GIP, OXM and PYY in various combinations has been shown to improve glycaemia, reduce food intake and decrease bodyweight in man[218,219,227,228]. Interestingly, continuous GIP infusion alone has been shown to produce a worsening of glycaemia in T2DM patients (potentially due to glucagon secretion)[229] which, in combination to its lack of glucoregulatory efficacy in T2DM

patients[20,21], suggests its therapeutic potential for T2DM may be more limited than that of GLP-1. However, research is now shifting toward developing compounds which can mimic the incredible weight loss and glucoregulatory effects mediated after bariatric surgery.

1.4 Incretin hormones

Understanding of the "incretin effect", observations of the reduction of incretin effect in T2DM patients[17,21] and improvement in post-bariatric surgery glycaemia contributed to by improved incretin responses has guided research toward generating pharmacotherapies which target the incretin response in T2DM patients. Theoretically, a drug which mimics the metabolic reprogramming that occurs after bariatric surgery could result in robust weight reduction and correction of hyperglycaemia and may even regenerate β cell mass.

1.4.1 GLP-1

1.4.1.1 GLP-1 physiology

GLP-1 is secreted post-prandially from L cells found predominantly in the ileum and colon of the GI tract[230]. GLP-1 is synthesised from preproglucagon (*Ppg*), a gene which is expressed in a number of tissues including the pancreatic α cell, L cells of the intestine and some neuronal populations in the hypothalamus[231,232]. Further processing by prohormone convertase 1/3 (PC1/3) in the L cell converts the prohormone into the 37 amino acid peptide GLP-1(1-37), and then further into GLP-1(7-37) and GLP-1(7-36NH_2) which is the primary active circulating form of GLP-1[233,234]. GLP-1(7-36NH_2) is rapidly cleaved in the circulation by dipeptidyl-peptidase-IV (DPP-IV) between the second and third amino acids, generating GLP-1(9-36NH_2), which is usually considered an inactive form of GLP-1 (although studies have suggested a biological role of GLP-1(9-36NH_2)[235,236]). GLP-1 is also cleaved by neutral endopeptidase 24.11 (NEP-24.11)[233]. The high sensitivity of GLP-1 to these enzymes, as well as rapid renal clearance, results in a circulating half-life for active GLP-1 of only 2 minutes in humans[237].

The GLP-1R is expressed in a number of tissues, including the pancreatic β cells and δ cells, stomach, GI tract, kidney, lungs, heart, various regions of the brain and peripheral nervous system[238-242]. The widespread expression of GLP-1R in both the periphery and central nervous system mediate a wide range of physiological responses. The origin of certain neuronally-mediated GLP-1 (or pharmacological GLP-1R agonists) is spatially distinct[242], introducing the possibility of activating specific populations of GLP-1R-expressing neurons by specialised pharmacological agonists, which will be discussed later. The GLP-1R is highly expressed on the pancreatic β cell, and peripheral infusions of GLP-1, as well as GLP-1R antagonists, exert profound effects on insulin secretion[243,244]. However, its short circulating half-life has raised the question of whether endogenous GLP-1 released by the gut persists at adequate concentrations to directly stimulate the β cell, or whether peri-intestinal vagal afferent neurons are required to sense GLP-1 and initiate a gut-brain-islet neural relay. Indeed, GLP-1R-expressing neurons are found in proximity to the L cell[245,246], with CNS circuitry linked to the islets[218], which may also provide a mechanism of GLP-1-mediated insulin secretion. Mice lacking vagal GLP-1R expression displayed impaired insulin secretion and hyperglycaemia, suggestive of neural regulation in GLP-1R-mediated insulin secretion[247]. However, specific activation of pancreatic GLP-1R populations are sufficient to control glucose metabolism[248], suggesting both peripheral and central processes are involved in GLP-1-mediated glucose control.

GLP-1 has a range of physiological effects which include stimulating insulin synthesis and secretion from the β cell, reducing gastric emptying, inducing satiety through activation of neuronal pathways, increasing insulin sensitivity and inhibiting glucagon secretion (See Figure 1.2 for and overview). Prolonged GLP-1 administration also increases β cell survival through reduced apoptosis, and increased β cell proliferation. The combined effect of these actions is to improve glycaemia and promote weight loss, suggesting the long-term effects of GLP-1 administration in the context of obesity and T2DM are advantageous. In T2DM, the secretion of GLP-1 in reduced which likely contributes to hyperglycaemia and β cell dysfunction[249]. Infusion of GLP-1 into T2DM patients can restore GLP-1-mediated effects including insulin secretion, insulin sensitivity and satiety, along with an increased weight loss compared to placebo[249,250]. Therefore, attempts to mimic the activity of GLP-1 are an exciting option for T2DM treatment.

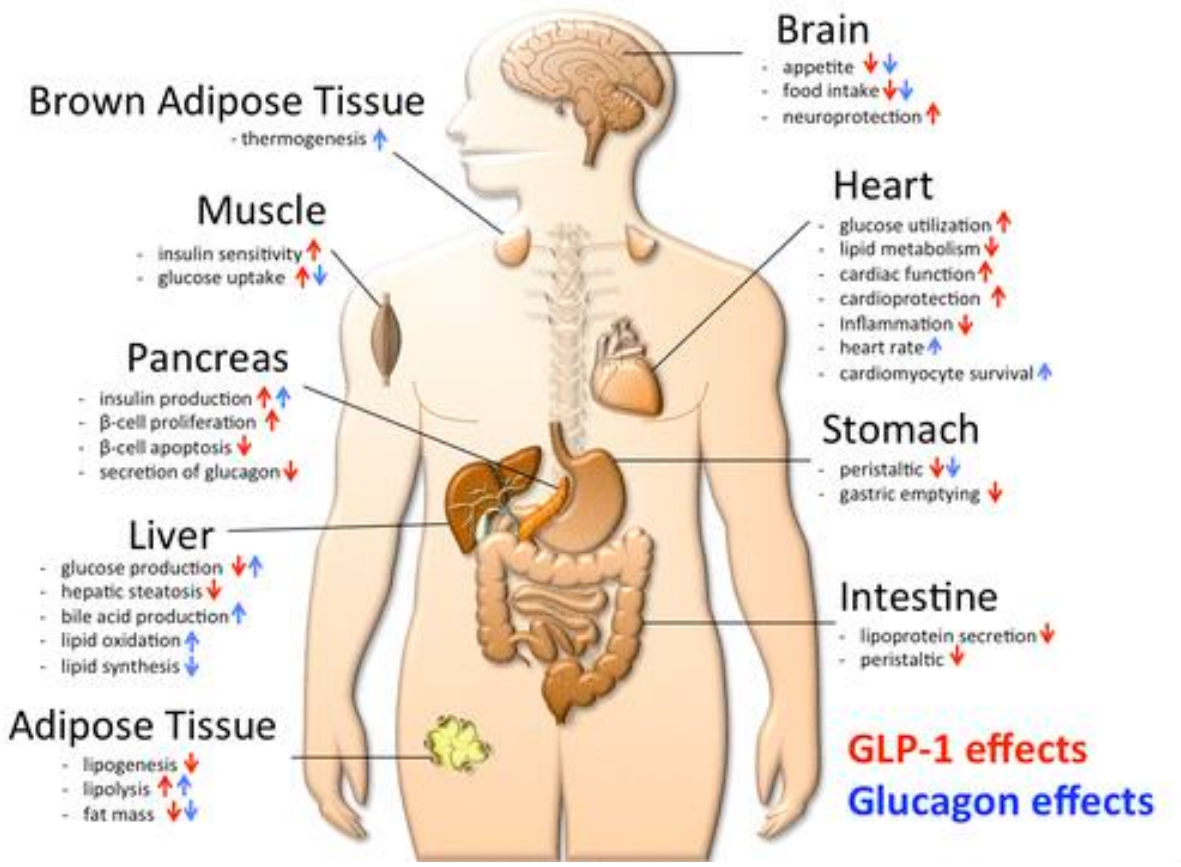

Figure 1.2 - Overview of the physiology of glucagon and glucagon-like peptide-1 (GLP-1).

Figure from *Müller et al.*[2]

1.4.1.2 GLP-1 receptor signalling

GLP-1R is a G protein-coupled receptor (GPCR). GPCRs are a major class of receptors, translating extracellular messages from diverse stimuli including light, small molecule metabolite, neurotransmitters and to hormones such as GLP-1. GPCRs have a conserved seven transmembrane (TM) α helical structure, hence their other name of seven transmembrane receptors (7TMRs), linked by intracellular and extracellular loops (ICL/ECL). GLP-1R are classified within the secretin, or class B, family of GPCRs, which also contain a large N terminal extracellular domain which contains an allosteric site responsible for ligand binding and specificity[251]. Ligands that activate class B receptors bind to both the large N terminal allosteric site as well as the orthosteric ligand binding domain within the 7TM pocket in a process termed “two-step activation”. For this reason, there are only a small number of small molecule agonists targeting the class B GPCRs, with the vast majority being large, peptide-based biomolecules.

When the GLP-1R is activated, conformational movement of the helices allows for the initiation of intracellular signalling cascades[252-255]. One important signalling cascade includes the reversible binding of various G proteins. Inactive G proteins are heterotrimers, formed of a Gα, β and γ subunit with guanosine-diphosphate (GDP) bound to the Gα subunit. Following ligand binding and receptor-conformational rearrangements, the G protein heterotrimer binds to a pocket within the receptor on the cytosolic side, resulting in exchange of bound GDP for a guanosine-triphosphate (GTP) molecule. This GTP-bound Gα subunit can then dissociate from the trimer and activate numerous downstream signalling cascades. There are different isoforms of Gα which affect different signalling pathways. For example, $G\alpha_s$ activates adenylate cyclase (AC) which catalyses the production of the secondary messenger cyclic adenosine monophosphate (cAMP), whereas $G\alpha_i$ inhibits AC, thus reducing cAMP accumulation. GLP-1R-mediated insulin secretion has been widely attributed to $G\alpha_s$-stimulated cAMP pathways, whereby cAMP activates protein kinase A (PKA) and exchange protein directly activated by cAMP-2 (Epac2). PKA phosphorylates the K_{ATP} channel, resulting in its closure which contributes to insulin secretion[256], although Epac2 has also been shown to inhibit the K_{ATP} channel[257,258]. Both PKA and Epac2 contribute to increased intracellular Ca^{2+} concentrations, through an interaction pathway involving phospholipase-C-ϵ (PLC-ϵ)[259], which has also been linked to enhanced insulin granule formation and exocytosis[260]. There is also evidence that GLP-1R activation of the Gβ/γ subunit initiates the phosphatidylinositol 3'-kinase (PI3K)/PKB pathway, which results in reduced β cell apoptosis by reducing ROS production and increased β cell growth by stimulating differentiation of pancreatic α cell progenitor cells[261-264]. In adipocytes, the GLP-1R agonist liraglutide inhibited the critical *de novo* lipogenesis enzyme fatty acid synthase (FASN) through a PKA-mediated mechanism, which lead to decreased adiposity in mice[265]. GLP-1R has also been shown to signal through $G\alpha_i$ (which inhibits AC) and $G\alpha_q$ (which mobilises intracellular Ca^{2+} through activation of diacylglycerol (DAG)), however the relevance of this is still unclear[266-268]. $G\alpha_q$ itself has been implicated in mechanisms of receptor internalisation and insulin secretion[269].

Activation of a GPCR is quickly followed by the recruitment of G protein receptor kinases (GRKs), which phosphorylate the receptor at the intracellular C-terminal tail allowing recruitment of β-arrestins to the receptor. The recruitment of β-arrestins to

GPCRs is classically associated with desensitisation of the receptor signal, as they sterically inhibit the interaction of G proteins with the GPCR binding site[270]. A further canonical role of β-arrestins is to promote internalisation of the receptor, through their action of scaffold proteins for clathrin and the clathrin adaptor molecule adaptor protein-2 (AP2) initiating clathrin-mediated receptor endocytosis[271,272]. This also occurs at the GLP-1R, which has been shown to be able to interact with both β-arrestin-1 and -2[254,273-275]. Ablation of β-arrestin recruitment to the GLP-1R by siRNA also leads to decreased receptor internalisation and prolongation of signalling at the receptor, suggesting β-arrestin-mediated internalisation reduces the signalling efficacy of the GLP-1R[276]. Two β-arrestin isoforms (-1 and -2) exist, with a degree of functional redundancy between the two: individual isoform knockout mice are normal at the phenotypic level, yet dual isoform knockout is embryonically lethal.

As well as their established role in terminating G protein signalling events, β-arrestins are now also being acknowledged as initiators of distinct, non-G protein-driven downstream signalling pathways. This is believed to result from their action as scaffold proteins for recruitment of different kinases[277,278]. Moreover, abundant evidence now exists that GPCR internalisation, including for GLP-1R, does not immediately lead to cessation of signalling. Rather, ERK1/2 and cAMP signalling can still occur in endosomes and appears to affect cytosolic phosphorylation rates of ERK1/2[279]. The GLP-1R associates with β-arrestin-1 which acts as a scaffold to initiate sustained ERK1/2 signalling, which have been associated with mediating GLP-1-mediated β cell survival[280] and insulin secretion[275]. Interestingly, β-arrestin-1 has not been shown to initiate GLP-1R internalisation, suggesting it may be implicated in G protein-dependant and -independent intracellular signalling mechanisms. Further studies have since emphasised the importance of β-arrestin signalling complexes as being vital to insulin secretion and sensitivity (albeit not specifically for GLP-1R) [281-284]. It is therefore clear that GLP-1R signalling is more complex than first imagined, with the effects of β-arrestin signalling potentially detrimental (by inducing desensitisation) and beneficial (by enhancing insulin secretion and sensitivity) to incretin biology.

1.4.1.3 GLP-1R agonists in T2DM and obesity

To translate the observed improvements in glycaemic control and weight loss after infusion of native GLP-1 in obese T2DM patients, efforts have been made to harness these beneficial effects in a practically deliverable drug formulation. Two primary approaches have been taken: 1) preventing the degradation of endogenous incretins, and 2) improving the pharmacokinetic and pharmacodynamic properties of GLP-1 through rational molecular optimisation.

Inhibitors of DPP-IV, the major degradative enzyme of GLP-1, are an approved drug class for the treatment of T2DM. The actions of DPP-IV cause the circulating half-life of active GLP-1(7-36NH_2) to be as low as a few minutes[237]. Therefore, inhibiting DPP-IV reduces the rate of incretin degradation, allowing for the glucoregulatory effects of GLP-1 to prevail for longer. There are four DPP-IV inhibitors currently on the market: sitagliptin, linagliptin, saxagliptin and alogliptin. As a monotherapy, DPP-IV inhibitors improve baseline glycaemia, improve islet function, and improve insulin sensitivity in T2DM patients compared to placebo[285-287] or sulphonylureas[288], and improves the glycaemic control when combined with metformin[289] or TZDs[290]. In addition to this, DPP-IV inhibitors are weight neutral, i.e. they do not lead to the disadvantageous weight gain that limits the therapeutic utility of insulin secretagogues such as TZDs and sulphonylureas[291].

The second strategy has been to develop long acting GLP-1R agonists with significantly greater circulatory half-lives than native GLP-1. In generally, pharmacological GLP-1R agonists display greater anti-hyperglycaemic properties than DPP-IV inhibitors[292-295], and are therefore regarded as the preferred approach to increasing GLP-1 activity in T2DM patients. The first pharmacokinetically-enhanced GLP-1R agonist, exendin-4 (Ex4), was identified by Dr John Eng in 1992 from the saliva of the Gila monster (*Heloderma suspectum*)[296] and shown to be an agonist at the GLP-1R[297]. Ex4 shares a 53% sequence homology with native GLP-1[296], with glycine at position 2 making Ex4 resistant to DPP-IV degradation[298]. The circulating half-life of Ex4 is reported to be 26 minutes to 2 hours in humans[299], depending on route of administration, making it therapeutically effective with twice-daily dosing. Consequently, exenatide and lixisenatide have been developed, which are structurally

based on Ex4[300]. A second approach has also been taken in which large biomolecules are attached to native GLP-1 to decrease the rate of renal clearance. These larger GLP-1R agonists include the recently licensed drugs liraglutide and semaglutide as well as albiglutide and dulaglutide. In general, the second option, adding large macromolecules to GLP-1 creates a longer lasting but less potent compound, whereas the opposite is true for amino acid-based GLP-1R agonists. For an in-depth discussion on GLP-1R agonist structures, see section 5.1.1.

The first licensed GLP-1R agonist, the twice-daily injectable exenatide (Byetta®) which is recombinantly-produced Ex4, was tested as an adjunct therapy to insulin or metformin. It led to improved glycaemic control by reducing HbA1c levels compared to placebo in patients with diabetes were on background medication[301,302]. Head-to-head studies indicate that exenatide leads to similar reductions in HbA1c as sulfonylureas and TZDs, but possesses the significant advantage of a lower risk of hypoglycaemia[301-304]. Therefore, since 2007 Byetta® has been licensed as a therapy for T2DM. Moreover, exenatide has evolved from a twice daily injection to a once-daily and once-weekly preparations, leading to a more effective GLP-1R agonist for treating T2DM. When tested directly against Byetta®, once weekly exenatide (Bydureon®) improves HbA1c levels by 0.4-0.7%[305,306]. Other GLP-1R agonists since have been shown to be even more effective than Bydureon® at improving basal glycaemia, with both liraglutide[307] and semaglutide[308] showing improved long term HbA1c reductions of 0.2-0.6% compared to Bydureon®. To date, semaglutide given as a once weekly injection, has shown to be the most effective GLP-1R agonist at improving HbA1c levels in T2DM patients, when compared to other GLP-1R agonists[308-312]. However, with variations in dosing, group size, demographics and length of studies, valid comparisons with all GLP-1R agonists are not possible outside of head-to-head comparisons.

When co-administered with metformin, twice-daily exenatide produced modest weight losses of 1-3kg *versus* placebo in human studies whilst displaying exceptional glycaemic improvements[301,313]. Bydureon® (once-weekly exenatide) produces between 2-4kg weight loss over six months to a year compared to placebo[306,314-316]. In Phase III clinical trials, the once-daily injectable liraglutide consistently resulted in a larger 5-10% weight loss between 20 weeks and 2 years compared to placebo, both

with lifestyle modifications included[317-322]. For this reason, liraglutide is marketed as an anti-obesity treatment as well as for diabetes. Semaglutide has also been shown to induce the greatest weight loss in diabetic obese individuals[309,323], even showing a greater weight loss *versus* liraglutide in non-diabetic[324] and diabetic obese patients[310,325]. As semaglutide is the first licensed oral GLP-1R agonist, this result is exciting as it suggests an oral drug, which is much preferred in patients compared to an injection, could provide superior weight loss compared to an injectable drug. However, it must be noted that in the O'Neil study, semaglutide was injected once daily rather than the once weekly recommended dose, which may affect circulating drug levels and nausea.

Despite their exciting clinical effects, GLP-1R agonists are commonly associated with side effects including nausea, diarrhoea, vomiting and GI disturbances[326]. These side effects reduce the potential efficacy of GLP-1R agonists, as dose finding studies have shown that enhanced glycaemic control is possible at doses beyond that given in standard therapeutic doses, suggesting the maximal therapeutic dose exceeds the tolerated dose. Nausea is associated with the passage of the GLP-1R agonist through the blood-brain barrier (BBB) into the brain[327]. This paradigm is supported by clinical observations of albiglutide, which displays low brain penetration due to its large size. Albiglutide is more readily tolerated by patients as it shows reduced rates of nausea; however, the lack of brain penetration also limits its ability to reduce food intake, leading to less impressive weight loss[328-330]. Interestingly, albiglutide scores lower on the patient satisfaction index than liraglutide, despite being better tolerated, due to the lesser weight loss acheived[327,328].

Other adverse effects of GLP-1R agonists have been suggested. Some of the most serious of these include pancreatitis and even pancreatic cancer. GLP-1R is expressed at low levels in the pancreatic exocrine tissue. Some clinical studies have shown marginally increased levels of circulating pancreatic enzymes in GLP-1R agonist-treated patients[331-333], whilst others have shown no difference[334-336]. Overall assessment of the available evidence does not support a causal relationship between GLP-1R agonist treatment and pancreatitis[337], and it is important to recognise that T2DM itself is a risk factor of pancreatitis[338], which has confounded interpretation of some studies.

1.4.2 Glucagon

1.4.2.1 Glucagon physiology

Glucagon, secreted primarily by the α cell, is classically associated as the counter-regulatory hormone to insulin, as it was reported in 1923 by Kimball and Murlin to cause hyperglycaemia. Like GLP-1, it is a product of the *Ppg* gene; however, it results from processing by PC2 in the pancreas, as opposed to PC1/3 for GLP-1 in the L cells[2]. Interestingly, glucagon shares significant sequence homology to GLP-1 and can therefore act as a weak agonist to the GLP-1R, albeit with 1000-fold lower potency[12]. Like most endogenously produced peptides, glucagon has a short circulating half-life between 4 and 7 minutes[339,340]. The most potent stimulant of glucagon secretion is hypoglycaemia, which is believed to induce glucagon secretion from the alpha cell in a manner similar to GSIS, however reduced adenosine triphosphate (ATP) caused by hypoglycaemia causes closing of the K_{ATP} channels in the α cell, as opposed to high intracellular ATP concentrations stimulating closure in the β cell[341]. A secondary hypothesis of hypoglycaemia-induced glucagon release is hypothalamic sensing of hypoglycaemia, which induces centrally-mediated secretion of glucagon from the α cell[342,343]. Insulin is a potent inhibitor of glucagon secretion, and it has been shown *in vitro* that glucotoxicity induces abnormal glucagon secretion in rodent α-like cells in the absence of physiological insulin signalling[344]. This suggests that glucotoxicity is implicated in T2DM by affecting the insulin/glucagon feedback mechanism.

Glucagon acts on its cognate receptor, the GCGR, to increase hepatic glucose production by increasing the activity and expression of enzymes involved in glycogenolysis and gluconeogenesis, as well as inhibiting glycolysis and glycogenesis[2]. The GCGR is predominantly expressed on the liver, but is also expressed to a lesser degree on pancreatic α and β cells, kidney, neurons and adipose tissue[345]. Acute responses to hypoglycaemia results in enhanced glycogenolysis, with little effect on gluconeogenesis[346]. However, upon prolonged fasting when glycogen stores are depleted, gluconeogenesis dominates the glucose-production drive[347]. With the introduction of glucagon detection assays, discovery of high circulating glucagon (hyperglucagonaemia) was noted in T2DM patients[348]. This produced two theories:

firstly, the bi-hormonal model of glucose homeostasis, where glucagon and insulin compete with each other to mediate normoglycaemic conditions. Secondly, it was thought that hyperglucagonaemia was a major determining factor for diabetes progression[349].

However, the biology of glucagon is much more complex than first believed. In glucagonoma patients, where α cell tumours result in hyperglucagonaemia, not all patients display diabetes mellitus or even hyperglycaemia[350-352]. Similarly, GCGR knockout animal models, or treatment with a GCGR-neutralising antibody or small molecule inhibitor does not result in extreme hypoglycaemia[353,354]. Finally, prolonged glucagon infusion into non-diabetic humans produces acute hyperglycaemia followed by euglycaemia, whereas diabetic humans without the compensatory insulin response showed hyperglycaemia[355,356]. This suggests that the bi-hormonal hypothesis does not adequately describe the control of blood glucose levels. Importantly, glucagonoma patients show excessive weight loss and extreme hypoaminoacidaemia (low circulating amino acid levels)[357]. It is now thought that glucagon acts through its hepatic receptor to increase expression of key enzymes involved in amino acid catabolism and subsequent gluconeogenesis[350,358-360]. In addition to this, glucagon stimulates ureagenesis, which is a key step in removing toxic ammonia produced from amino acid catabolism[361,362]. Through these mechanisms, glucagon increases the flux of amino acids into the liver as a means of gluconeogenic substrates. Whether an increased supply of amino acids, through diet or therapy, can be used to counteract the hypoaminoacidaemia observed from chronic GCGR stimulation would be interesting to observe, as it may present a means of ablating muscle wasting which could hamper GCGR agonist therapies.

The ability of glucagon to contribute sources of energy is not limited just to glucose homeostasis. In humans, periods of extended fasting where glucose availability is compromised, glucagon also stimulates lipid metabolism to serve as an alternative fuel source. In hepatocytes, glucagon stimulates lipolysis (the breakdown of complex fats into fatty acids) from stored triglycerides by phosphorylating key lipolytic hormones[363] in parallel to an inhibition of *de novo* fatty acid synthesis[364]. In addition to stimulating FFA production, glucagon stimulates ketogenesis and β oxidation of FFA in hepatocytes, a process which results in the breakdown of FFA, producing ATP as

an energy source[365]. However, in humans the lipolytic effect of glucagon was minimal at physiological levels[366-368], and effects at supra-physiological levels were ablated by concurrent insulin administration[369-372], suggesting in humans any lipolytic effect is only relevant when insulin levels are low (for example, during fasting).

In humans, glucagon may cause a reduction in food intake[373-376], however this may be dose- or administration-related. Glucagon is capable of penetrating the blood brain barrier to act on hypothalamic neurons to induce food intake reduction[377,378]. Interestingly, infusion of glucagon into the hepatic portal vein, a site of higher post-prandial glucagon concentrations also leads to satiety[379], suggesting vagal signalling may mediate glucagon-mediated satiety. A role of glucagon in increasing energy expenditure is also apparent, as pair fed rats given glucagon lost more weight than placebo-treated rats[380]. Similarly, humans that are infused with glucagon display increased oxygen consumption, in line with an increase in energy expenditure[227,228,381,382]. One possible mechanism for this could be non-shivering thermogenesis activation of brown adipose tissue (BAT), as rodent studies showed increased oxygen consumption and thermogenesis when glucagon was administered[383,384], and mice deficient in glucagon peptide production display reduced thermogenic abilities in cold conditions[385]. Levels of UCP-1, a key mitochondrial enzyme involved in the thermogenic response, increase in rodent BAT after glucagon administration[385], suggesting a mechanism of increased energy utilisation by thermogenesis using UCP-1 in BAT. In humans, glucagon levels rise upon exposure to cold[386], suggesting an adaptive glucagon-mediated response to cold-induced thermogenesis. However, human studies have cast doubt on a major role for BAT in GCGR-mediated energy expenditure[381,387]. An alternative theory is that glucagon increases the “browning” of white adipose tissue (WAT), allowing for a greater extent of BAT thermogenesis[388].

Glucagon is also a potent stimulant of insulin secretion. Indeed, mixed-nutrient meals induce a rise in circulating glucagon in healthy humans, suggesting a physiological role of glucagon in the post-prandial state[389]. The GCGR is present on the pancreatic β cell[390], and in vitro perfusions of pancreata suggest that glucagon is able to stimulate insulin secretion from the β cells, in conditions of high glucose conditions[12,391,392]. Interestingly, owing to its weak affinity at the GLP-1R, glucagon is able to instigate

insulin secretion by activating both the GCGR and GLP-1R[12]. In addition to this, overexpression of the GCGR in mouse β cells improves glucose control through increased β cell proliferation and insulin production[393], further suggesting a functional role for GCGR activation in the overall insulin secretory response. When human islet cells were treated with the GCGR antagonist des-His1-[Glu9]-glucagon-amide, insulin secretion was reduced in hyperglycaemic conditions[390]. Owing to this, Kim *et al.* investigated the effect of a GCGR agonist on insulin secretion and sensitivity[394]. Here, treatment of a GCGR agonist prior to an IPGTT resulted in improved insulin sensitivity compared to vehicle, suggesting a positive insulinotropic ability of GCGR activation. As GCGR activation is typically associated with hyperglycaemia, these findings suggest that utilising GCGR agonism in a therapeutic setting could produce beneficial insulinotropic effects in the post-prandial setting.

1.4.2.2 Glucagon receptor signalling

Like the GLP-1R, the GCGR is a class B GPCR. Activation of hepatic GCGR leads to the activation of $G\alpha_s$, activating AC to produce cAMP which in turn activates PKA. In the liver, activated PKA plays a number of roles critical to glucagon physiology. Activated PKA can migrate to the nucleus to activate TORC2 which enhances the expression of key gluconeogenic enzymes including phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase)[2,395,396]. Activated PKA also leads to activation of glycogen phosphorylase (GP) which promotes glycogenolysis and production of glucose-6-phosphate (G6P), which is a key substrate of gluconeogenesis. PKA/TORC2 signalling has also been linked to glucagon mediated effects on hepatic β oxidation of lipids[397] and inhibits lipogenesis by inhibiting the key enzyme acetyl-coenzyme-A carboxylase (ACC)[363].

Glucagon can also signal through coupling to $G\alpha_q$ [398-400], and a GCGR agonist which specifically couples to the Gαq pathway has been shown to initiate hepatic glycogenolysis, gluconeogenesis and ureagenesis[399]. This could be through regulation of the hepatic TORC2 regulator CREB-regulated transcription coactivator 2 (CRTC2)[2,401]. PKC, which is activated through the $G\alpha_q$ signalling cascade, but not PKA, has also been shown to mediate glucagon-mediated GCGR desensitisation[402] and internalisation[403], therefore $G\alpha_q$ signalling at the GCGR may also contribute to

either self-phosphorylation or activation of other GRKs to cause desensitisation[402] which causes the recruitment of β-arrestin to the receptor for internalisation[403].

The role of β-arrestin recruitment in GCGR responses is less understood than for GLP-1R signalling. Like the GLP-1R, β-arrestin recruitment is a potent regulator of the receptor, resulting in GCGR desensitisation and internalisation[283,403]. When β-arrestin-2 is knocked out of the murine liver, hepatic GCGR cAMP signalling is increased and hyperglycaemia ensues, suggesting β-arrestin-2 could mediate GCGR desensitisation and internalisation[283]. Interestingly, in this study, hepatic β-arrestin-1 knockout did not affect glucose homeostasis, which suggests its roles are distinct from receptor desensitisation and internalisation. B-arrestin-1 does associate with the GCGR, and both β-arrestin isoforms are recruited to the receptor when internalised[403]. The combination of the Zhu and Krilov studies therefore suggest that β-arrestin-2 is essential for GCGR internalisation (as knock out of it resulted in attenuated internalisation), and further recruitment of β-arrestin-1 enables signalling to other intracellular pathways. Whether this indicates β-arrestin-1 primarily acts as a scaffold for non-$G\alpha_s$-mediated pathways, or it plays no role in GCGR pharmacology, is unknown.

1.4.2.3 Targeting the glucagon receptor in T2DM and obesity

The revelation that circulating glucagon levels are elevated in T2DM patients led to research into antagonists of the GCGR as a route of treatment for T2DM. Additionally, glucagon is used as a therapy against hypoglycaemia (such as that from insulin overdose), therefore blocking the activity of glucagon could theoretically result in eu-/hypoglycaemia. Whilst there has been success in improving glucose tolerance in animal models of T2DM[404-406], no GCGR antagonist has progressed to the clinic as investigational GCGR antagonists result in hepatic steatosis and worsened circulating lipid profiles[407,408].

Direct infusion of glucagon into T2DM humans is associated with acute hyperglycaemia[227,228], which limits the ability of a GCGR mono-agonist to be used as a therapy for any patient with T2DM. However, addition of GLP-1R agonism corrects hyperglycaemia whilst maintaining beneficial GCGR-mediated effects. Intriguingly, it

may be possible to use GCGR agonists to promote euglycaemia. In rodent studies, injection with a GCGR agonist resulted in acute hyperglycaemia (as expected from hepatic glycogenolysis) followed by prolonged *improved* glucose tolerance and insulin sensitivity[394]. Agonists that have concomitant GCGR activity, such as GLP-1R/GCGR agonists or GLP-1R/GIPR/GCGR tri-agonists, similarly display enhanced glucoregulatory abilities in patients with T2DM (see section 1.4.3.3), suggesting activating the GCGR in the context of hyperglycaemia may, in fact, be beneficial.

The beneficial effects of glucagon therapy extend to its effects on lipid metabolism, satiety and energy expenditure[2]. In humans, glucagon administration enhances energy expenditure[227,228,381,382] leading to weight loss[374,382]. Co-infusion studies with GLP-1R agonism also results in a reduction in food intake alongside increases in energy expenditure[227,228,381], making GCGR agonists an attractive component of a combination anti-obesity drug approach. Alongside its beneficial effects on body weight, glucagon infusion may modulate lipid metabolism in diabetic humans. When pharmacological levels of glucagon were infused into non-diabetic or diabetic humans, diabetic patients displayed enhanced lipolysis and ketogenesis[371], however the site of lipolysis (either adipose or hepatic) was not investigated. This effect is not observed at physiological levels of glucagon[409]. Interestingly, adipose-derived lipolysis (which results in increased circulating FFA which could be harmful to hepatic health) is inhibited by insulin, but insulin-mediated inhibition of hepatic lipolysis has not been specifically noted. Therefore, a combination of glucagon and insulin could improve hepatic lipid levels without detrimental lipolysis deriving from the adipocyte. More in depth investigation in rodents show that after glucagon administration, hepatic triglyceride accumulation reduces and an improvement in liver health is observed[397,410-412]. Finally, impaired glucagon signalling is observed in patients with the obesity-related disease non-alcoholic fatty liver disease (NAFLD)[413-415], which may contribute to triglyceride accumulation through a reduction in lipid metabolism. Therefore, pharmacological stimulation of GCGR signalling may help to reverse fatty liver.

As well as its own metabolic effects, glucagon has been used as a means to deliver additional metabolic modulators to the liver. Finan *et al.* conjugated glucagon to thyroid hormone T3 with the aim of achieving a form of “liver-specific hyperthyroidism”[416].

Endogenous T3 shows favourable effects on energy expenditure, fatty acid oxidation and cholesterol metabolism through its actions on the liver[417,418], but serious side effects such as myocardial arrythmias and osteoporosis related to myocardial and bone thyroid hormone receptor, limit its use as a treatment[419]. In mice, treatment with the glucagon-T3 conjugate resulted in improved glucose tolerance, improved insulin sensitivity, weight loss and lower hepatic lipid and cholesterol levels without leading to side effects by actions on other tissues[416].

Therefore, the therapeutic benefits of GCGR are striking and, due to the early discovery that it induces hyperglycaemia, not fully understood. Integration of GCGR agonism into therapies could enhance therapeutic outcomes related to glucose homeostasis, insulin utility, body weight and fatty liver.

1.4.3 Oxyntomodulin

1.4.3.1 Oxyntomodulin physiology

Another incretin that is observed at higher circulating levels post-bariatric surgery is OXM. Like GLP-1 and glucagon, it derives from the *Ppg* gene and is secreted post-prandially from L cells[420]. Structurally, OXM is composed of the 29 amino acids of glucagon, plus an eight amino acid tail, called intervening peptide-1(IP-1). It shares close sequence homology with both GLP-1 and glucagon, therefore acts as an agonist at both receptors, albeit with reduced potency compared to the endogenous ligands[421,422]. Like GLP-1 and glucagon, it is rapidly degraded by DPP-IV and renal clearance once secreted into the circulation, with a circulating half-life of approximately 10-12 minutes[423,424].

Physiologically, as it is secreted from the L cells with GLP-1 it is thought to share a similar physiological role to GLP-1. OXM is secreted post-prandially and is therefore believed to control glycaemia through its insulinotropic abilities on the GLP-1R[425], with similar unresolved questions regarding a direct β cell *versus* neutrally-mediated mechanism of action. Acutely, lean-healthy, obese-healthy and obese-T2DM humans administered with physiological post-prandial levels of OXM all display enhanced glucoregulatory abilities and enhanced insulin secretion[426,427]. OXM also reduces food

intake at physiological levels without affecting gastric emptying, unlike GLP-1, in healthy humans[426,428], however the lack of effect on gastric emptying has been questioned[376]. Another mechanism by which oxyntomodulin may increase satiety is by inhibiting the orexigenic hormone ghrelin[428,429], however again this has been refuted[430,431]. Therefore, the exact mechanism by which OXM induces weight loss is unknown.

Mice injected with oxyntomodulin or GLP-1 show differential neuronal activation patterns, suggesting differences between the two hormones in their ability to modulate centrally-mediated GLP-1R activities such as satiety and insulin secretion, however this has not been confirmed in humans[432,433]. Finally, OXM has been shown in GLP-1R or GCGR knockout mice to increase the heart rate though a GCGR-dependant mechanism and reduces body temperature via a GLP-1R-mediated mechanism[434]. No cardiovascular effects have been observed in humans infused with physiological levels of post-prandial OXM[426], meaning that these effects might only occur at pharmacological doses.

1.4.3.2 GLP-1R/GCGR agonists

Integrating the activity of two biological molecules in one therapy may provide a beneficial treatment strategy compared to monotherapy. In the case of GLP-1R mono-agonists, their therapeutic use is limited by its nauseating and GI disturbances, and glucagon agonism by its excessive hyperglycaemia and amino acid catabolism leading to secondary muscle-wasting. Therefore, activating two or even three incretin receptors simultaneously may additively produce a more effective therapy whilst bypassing side effects associated with activation of the individual receptor by allowing lower dosing of each individual component[416,435].

Oxyntomodulin is a naturally-produced dual agonist at both the GLP-1R and GCGR agonist which is found at higher circulating concentrations after bariatric surgery[221]. Through its actions at the GLP-1R, it is thought to enhance insulin secretion and satiety, both of which are enhanced after bariatric surgery. Alongside the primarily GLP-1R-dependant reduction in food intake, GCGR activation contributes to weight loss through increased energy expenditure. Whilst GCGR activation results in

hyperglycaemia, this could be offset by the insulinotropic abilities of GLP-1R signalling at the β cell. When physiological levels of OXM alone are infused chronically into obese humans, greater weight loss is observed than with GLP-1R activation alone[424], which is attributed to the GCGR activity[227,228,424]. Alongside the improved weight loss, OXM infusion improves insulinotropic and glucoregulatory abilities to that of GLP-1R agonism alone[227]. Similarly, infusion of both GLP-1 and glucagon shows superior effects on weight loss and food intake compared to the individual hormones, without affecting glucose tolerance[228]. This suggests that addition of GLP-1 can mask hyperglycaemia associated with glucagon mediated gluconeogenesis, which could allow the beneficial effects of both hormones to be combined to improve the treatment of T2DM and obesity.

Therefore, attempts have been made to produce dual GLP-1R/GCGR peptide agonists based on the structure of OXM, but with enhanced resistance to rapid DPP-IV-mediated degradation which limits the circulating half-life of OXM to 10-12 minutes[423,424]. The first example was developed by DiMarchi & Tschöp, who altered amino acids within OXM and added a polyethylene glycol (PEG) side chain to produce a PK-enhanced dual agonist[436]. In rodents this resulted in exceptional weight loss *versus* GLP-1R agonism alone with equal improvements in glucose handling[436]. A second GLP-1R/GCGR dual agonist, initially designed by Alessandro Pocai and later developed by Merck (MK-8521), is based on OXM with protective amino acid substitutions and a cholesterol residue added at the end[437]. Preliminary studies in rodents showed similar impressive weight loss and glucoregulatory improvements as the *Day et al.* molecule, attributed to signalling at both receptors, and superior weight loss *versus* a GLP-1R-selective compound[437]. Preliminary phase II data in obese T2DM patients suggests a reduction in Hba1c levels of 0.6% after 12 weeks compared to placebo, whereas liraglutide reduced HbA1c by 1%[438]. Liraglutide was, however, given at a six times higher dose which likely explains the improved HbA1c results. MK-8521 treatment resulted in a weight loss of 1.7kg after 12 weeks, whereas liraglutide at a six times greater dose achieved a weight loss of 1.6kg[438]. If administered at a higher dose, to match the improvement in HbA1c, the weight loss difference would likely be more pronounced. SAR425899, a Sanofi-developed dual GLP-1R/GCGR agonist, has also been developed based and is an acylated form of an Ex4/glucagon hybrid. It is currently in phase II human trials, and has shown weight loss of 5.3-5.5kg

after 28 days of treatment in two cohorts of overweight/obese patients who were either metabolically healthy or T2DM[439]. Similarly, SAR425899 reduced HbA1c levels by 0.5% and improved β cell function and glucose tolerance in both groups compared to placebo[439-441]. Finally, cotadutide (MEDI0382), was designed by combining the glucagon backbone with key GLP-1R-activating amino acid residues with a palmitoylation site at position 10[442]. Displaying similar improvements in body weight reduction and glycaemic control compared to a GLP-1R-selective agonist in mice[442], MEDI0382 is in phase II of clinical trials. Initial phase II data shows that 6 weeks of cotadutide results in an improvement in post-prandial glucose tolerance, a reduction in HbA1c by 0.3% and a weight reduction of 2.1kg *versus* placebo[443]. In addition to this, it significantly improved hepatic lipid removal, correlating with improved weight loss[444]. Further dual GLP-1R/GCGR agonists are in various stages of development and their progress will be equally exciting to observe[424,445-447]. It will be crucial in future to compare the relative effects of new GLP-1R/GCGR dual agonists *versus* class-leading GLP-1R mono-agonists, such as semaglutide, in obese T2DM patients.

Despite the exciting weight loss effects and improvements to β cell function and glucose tolerance, nausea and other GI effects remain an issue for all the treatments discussed here. Increasing the “therapeutic window” of these agents is a necessary step to optimise the therapeutic potential of this drug class.

1.4.3.3 Other multi-incretin therapies

Examples of other dual incretin therapies include the incorporation of GLP-1R and GIP agonism. Whilst the therapeutic potential of GLP-1R agonism has been clearly demonstrated, the benefits of GIPR agonism have been harder to identify. Whilst GIP is thought to be a major incretin under physiological conditions, the anti-hyperglycaemic properties of GIP are markedly reduced in T2DM patients[20,21], and there are studies which suggest GIP may even contribute to an increase in adiposity[448,449]. This has resulted in perhaps paradoxical interest in GIPR antagonists as an anti-obesity strategy[450,451]. On the other hand, it has also been shown that GIP peptide over-expression leads to improved glucose control, hepatic lipid profiles and insulin secretion without changes in weight gain[452]. This data is backed up in longitudinal studies in rodents which suggest an improvement in β cell function and

glucose control[453,454]. The re-emergence of GIP as a candidate anti-diabetes therapy has led to GIPR/GLP-1R dual agonists being developed[455,456]. Pre-clinical rodent studies validate GIPR/GLP-1R dual agonism as a means to achieve greater weight loss, improved glycaemic control and reduced GI toxicity *versus* GLP-1R agonism alone[456,457]. In humans pre-clinical studies, it reduced HbA1c by up to 0.3% after 2 weeks[458] or 0.9% over 6 weeks in T2DM patients *versus* placebo[456]. In the final study, a modest weight loss of 1.8kg was also observed for patients on drug *versus* placebo[459]. A second GLP-1R/GIPR agonist tirzepatide (LY3298176), which is a once-weekly injectable, has also been developed and is in phase II trials. High-dose (10-15mg) tirzepatide improved glucose tolerance and reduced HbA1c levels in patients with T2DM by 0.6% by 4 weeks[455], 2.0-2.2% by 12 weeks[460] and 1.9% by 26 weeks[461]. Compared to placebo, high dose tirzepatide also reduced body weight by 4.5kg by 4 weeks[455], 5.0-5.2kg by 12 weeks[460] and 10.9kg by 26 weeks in obese T2DM patients. Interestingly, it also increased weight loss by 2.7-3.2kg by 4 weeks[455] and 8.6kg by 26 weeks[461], compared to the GLP-1R agonist dulaglutide. It is important to highlight here that the dose of tirzepatide was five-fold greater than for dulaglutide in this study, and also that the GLP-1R signalling potency of tirzepatide is also five-fold weaker than dulaglutide; therefore, equivalent GLP-1R might be achieved, making it difficult to conclude whether GIPR action contributes significantly to its overall effect in humans. Tirzepatide modulates lipid and carbohydrate metabolism in differentiated human adipocytes through a GIPR-mediated mechanism, which may explain the greater weight loss[462]. However, the incidence of nausea and GI side effects was present in approximately 50% of the patients on high dose tirzepatide, limiting the extent of further weight loss[460,461]. Tirzepatide also reduced gastric emptying rates, through a GLP-1R-mediated mechanism, which likely contributes to satiety and weight loss[463].

A monomeric tri-agonist acting at the GLP-1R, GCGR and GIPR has also been developed[464]. In addition to the insulinotropic effects of GLP-1R signalling and the energy expenditure effects of GCGR signalling, the additional GIPR agonism adds a further insulinotropic element as well as the putative beneficial effects of GIPR agonists on lipid metabolism. In mice, the Finan *et al.* tri-agonist displayed a 20-day weight loss of 27%, primarily relating to fat mass loss[464,465], and improved steatohepatitis[465]. Similar results were observed in the Choi *et al.* agonist (HM15211), where the weight loss observed is greater than that of liraglutide alone[466]. Whilst the

apparently improved therapeutic potential of these novel molecules is purported to relate to its actions all three receptors[464], no side-by-side studies have been performed with either a dual GLP-1R/GCGR or GLP-1R/GIPR agonist to observe the improvement attained by tri- as opposed to dual-agonism.

Other combinations of incretin hormones, such as GLP-1, oxyntomodulin and peptide YY (PYY, another anorectic hormone), have also been trialled. The latter combination, named "GOP", has been infused in overweight patients with prediabetes/T2DM. All three hormones are observed at much higher levels post-bariatric surgery, therefore GOP infusions closely mimic the post-surgery incretin state[219], albeit with the elevated hormones being sustained over many hours, unlike in the physiological setting. Here, GOP produced a 28-day weight loss of 4.4kg which, whilst slightly less than gastric bypass comparators, also showed superior glucose tolerance than bypass patients[218]. The weight loss observed was likely a combination of reduced food intake and increased energy expenditure[219].

It is therefore clear that incretin/hormone multi-agonist therapy is a successful method of a) improving glucose tolerance and β cell function and, b) causing greater weight loss than through GLP-1R agonism alone. OXM, which benefits from GLP-1R-mediated glucose control and satiety in conjunction with GCGR agonism to increase weight loss through energy expenditure is a prototypical example on which a number of synthetic pharmacological agonists have been based. However, the majority of these multi-incretin therapies are still limited by similar side effects to those of individual hormone mono-agonism. Therefore, a means of bypassing the tolerability limitations, whilst maintaining their enhanced therapeutic actions, is a potential "golden-ticket" for tackling the diabesity crisis discussed in this introduction.

1.5 Biased Signalling

1.5.1 Introduction

GPCRs signal through a wide range of G protein-, β-arrestin- and non-canonical signalling pathways. The transition from receptor activation to receptor signalling at all

GPCRs has traditionally described a “two-state” model of receptor activation, where ligand binding resulted in an “active conformation” of the receptor and all intracellular pathways were activated[467]. Through this method, there a range of monotonic signalling profiles available, based around the potency and efficacy of the ligand to the GPCR. However, there has been a paradigm shift in our understanding of GPCR pharmacology over the past 30 years, resulting in new knowledge of how GPCR signalling can be therapeutically utilised. It is now accepted that agonists binding to a receptor can activate selective downstream signalling pathways over others by stabilising the receptor in a certain conformation, termed “biased agonism”, “biased signalling”, “functional selectivity” or “selective signalling”[468-470]. X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy and cryo-electron microscopy studies have confirmed that activated receptors can form various conformations, which leads to unique recruitment profiles for intracellular signalling molecules[471-473]. A ligand which can induce a conformation leading to the selective pathway activation is therefore termed a “biased agonist” (Figure 1.3).

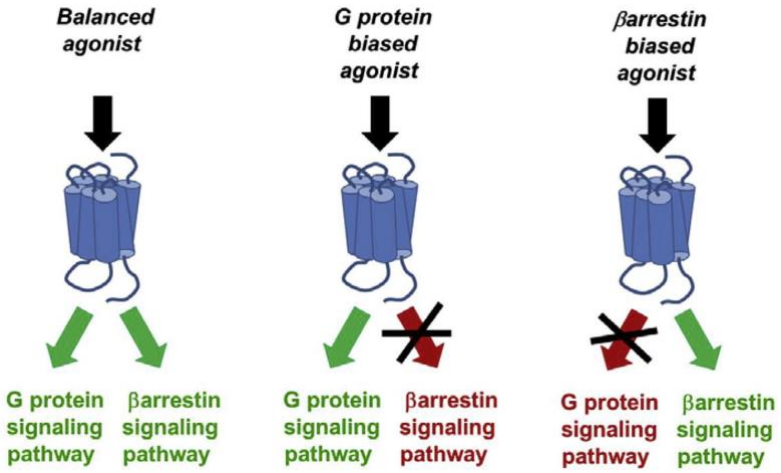

Figure 1.3 - Schematic of biased signalling

Figure taken from Rankovic *et al.*[1]

To confirm a ligand as being “biased” or “selective”, it must be compared against a reference ligand[3]. This is because endogenous ligands rarely activate all intracellular signalling pathways with equal potency and efficacy, therefore comparisons must be made on an individual basis. Adding to this complexity, activation of a given pathway can vary between cell-type or assay used, referred to as system-bias[469,474]. Here, cell-specific expression levels of signal transducer molecules (such as G proteins, AC,

DAG, GRKs, β-arrestins) or the target receptors can affect the coupling of the ligand-receptor conformation to the potency and efficacy of recruiting the signal transducers[474,475]. Another factor which can affect the observed bias is the sensitivity of the assay to visualise a response, termed "observational bias"[469]. Observational bias affects the potency and efficacy of the stimulus-response relationship, and is affected by reaction conditions such as temperature, time and reagents used. It is known that observational bias is affected by temporal properties of GPCR signalling[279], therefore it is critical that experimental conditions are carefully considered and maintained to avoid confounding effects of observational bias on the results.

To avoid inappropriate identification of a biased agonist, comparisons between two or more ligands should be preferably carried out using the following conditions:

1) Using the same cell line to avoid "system bias".
2) Use equal testing conditions (time, temperature) to avoid "observational bias".
3) Using matched assays to avoid differences in response amplitude or potency.

If these stringent conditions are maintained, the results gained can aid in the validity of translating interesting *in vitro* signalling data into an *in vivo* response.

Once experimental parameters are normalised, quantification of bias should be performed to allow comparisons between agonists. Using potency (pEC_{50} values) or efficacy (E_{max} values) alone discriminates against the other pharmacological parameter, whereas in many cases the two are not linked. Therefore, using a quantitative scale that incorporates agonist potency and efficacy into a single parameter would be preferable. The most commonly used model for quantifying bias is the modified operational model of agonism[476]. Here, agonist efficacy is defined by the parameter τ, which is the receptor density of the system (R_t) divided by the intrinsic efficacy of the agonist to activate a particular cellular response pathway (K_E), therefore $\tau=R_t/K_E$. A ligand's ability to activate a given intracellular pathway is also dependant on its intrinsic affinity (K_A). Both efficacy (τ) and affinity (K_A) are combined into the form $\log(\tau/K_A)$ to account for affinity and efficacy in a system-independent manner[469,476]. Two log(τ\KA) values are then compared to produce the comparative form

Δlog(tau/K_A). Using system-independent measures, such as log(τ/K_A) are useful when comparing agonist bias across different cell types or animal models which will have different receptor and transducer quantities. Other methods are available, such as the relative activity scale (ΔlogEC_{50}/E_{max}) which is a simpler method using the estimated values EC_{50} and E_{max}[477,478], or the "competitive scale" for cases of extremely partial biased agonists where pharmacological parameters are inaccurately calculated[479]. Reviews are available which discuss when different quantification models can be used[3,480-482].

1.5.2 Therapeutic Applications of Biased Agonists

The claimed therapeutic utility of biased signalling is to selectively activate intracellular signalling cascades associated with the therapeutic efficacy of a ligand, whilst reducing signalling to pathways which may be associated with side effects. This expands the repertoire of ligands available to therapeutically target GPCRs and could improve the therapeutic efficiency of activating a GPCR by widening the therapeutic window.

Numerous examples exist of biased agonists being used to improve the therapeutic utility of agonists to a GPCR. Canonical agonists at the μ-opioid receptor (MOR) (such as morphine and fentanyl) are an important drug class for providing analgesia in cases of chronic pain or inflammatory pain. However, their clinical usefulness is limited by the side effect profile, including respiratory depression, dependency and GI effects[483]. Interestingly, many of the side effects have been associated with β-arrestin signalling[484-487]. Therefore design of G protein-biased drugs, i.e. compounds which show reduced β-arrestin recruitment for the same degree of G protein activation, should theoretically minimise adverse effects associated with MOR agonists whilst also simultaneously prolonging signalling by reducing receptor desensitisation[488]. In mice which have β-arrestin-2 knocked out, or RNA-interference, MOR agonist tolerance, respiratory depression and GI effects are decreased and treatment efficacy is increased[485,488,489]. Therefore, G protein-biased agonists have been developed which show improved therapeutic efficacy compared to morphine in mice. The first G protein-biased agonist published was oliceridine (TRV130), which showed equivalent analgesic properties as morphine but reduced side effects in rodents[490]. In phase III

trials, oliceridine has shown similar improvements in analgesia in humans *versus* morphine and/or a reduced incidence of adverse effects[491-493]. Whether these short-term studies translate into longer term benefits remains to be seen. Interestingly, oliceridine was initially refused Food and Drug Administration (FDA) approval in 2018 due to other associated side effects relating to cardiac dysrhythmia. However, proof of principle has been provided that biased signalling can be leveraged to improve the therapeutic window of MOR agonists in humans. This has led to further discovery and development of other biased MOR agonists with better pre-clinical efficacy[494].

Similar approaches have been used to investigate examples of biased agonists at other therapeutically relevant GPCRs. A list of some examples where biased agonists have displayed improved *in vivo* efficacy or reduced adverse effects include G protein-biased κ-opioid receptor (KOR) agonists for treatment of analgesia[495], G protein-biased δ-opioid receptor (DOR) agonists for the treatment of chronic pain[496], G protein-biased serotonin type 2B (5-HT_{2B}) receptor agonists for the treatment of obesity[497], G protein-biased 5-HT1A agonists for anxiety and depression[498], β-arrestin-biased angiotensin II receptor type 1 (ATIIR) agonists for the treatment of hypertension[499], β-arrestin-biased CXCR3 agonists to increase the inflammatory response[500] and G protein-biased M2 muscarinic receptor (M2R) for the treatment of pain[501]. Therefore, the potential benefits that biased signalling can offer has been demonstrated for a number of therapeutically relevant target receptors. Nevertheless, it should be emphasised that rational biased ligand design requires an understanding of the contribution of each individual signalling pathway to the overall physiology of a given receptor.

1.5.3 Biased Signalling at the GLP-1R and GCGR

The application of biased to the GLP-1R has been explored, initially by comparing signalling profiles of endogenous GLP-1R ligands. Firstly, OXM was initially shown to differentially activate the cAMP and β-arrestin pathways at the GLP-1R compared to GLP-1, the first suggestion of endogenous bias at the receptor[422]. Later, both OXM and the GLP-1R agonist Ex4 were shown to selectively activate β-arrestin recruitment compared to GLP-1, whilst OXM more readily activates ERK1/2 phosphorylation compared to both GLP-1 and Ex4[254,279]. The increased β-arrestin and ERK1/2

coupling of OXM links to its ability to induce greater cytosolic ERK1/2 phosphorylation than GLP-1[279]. This further suggests OXM may engender different intracellular responses to GLP-1R activation than GLP-1.

Biased GLP-1R agonists have also been developed by incorporating amino acid substitutions into a parent agonist sequence such as Ex4. Such sequence modifications lead to altered interactions between the ligand and the transmembrane and extracellular portions of the receptor, both of which modulate signal bias[254,279]. The first published example of a synthetically-designed biased GLP-1R agonist was "P5"[502]. P5 is based on Ex4, but had the first 8 amino acids exchanged for a separate sequence of 7 amino acids, producing a peptide which displays comparable cAMP accumulation to Ex4, but with diminished β-arrestin recruitment[502]. It has since been shown that this change in signal bias is the result of differences in orientation of the extracellular loop 3 and interactions in the transmembrane region of the receptor[503]. P5 was shown to be more effective in preclinical *in vivo* models of diabetes, leading to better improvements in glycaemia than Ex4 at the same dose, highlighting its therapeutic utility[502]. P5 appeared to be less insulinotropic than Ex4, however the mechanism behind this finding was not confirmed.

Modification of N-terminal ligand amino acids of Ex4 also resulted in the development of another G protein-biased GLP-1R agonist, exendin-Phe1 (Ex-phe1), in which the first amino acid of Ex4, histidine (His), was substituted for phenylalanine (Phe)[276]. The resulting peptide showed reduced potency at the GLP-1R for cAMP accumulation but near complete abolishment of β-arrestin recruitment. Associated with the lack of β-arrestin recruitment, GLP-1R internalisation was also reduced, and recycling rate increased, compared to Ex4 treatment, resulting in greater *in vitro* insulin secretion from rat and mouse β cell lines[276]. *In vivo*, Ex-phe1 was equipotent to Ex4 for acute glucoregulatory abilities in mice (assessed by IPGTT), however after 4- and 8-hours, the glucoregulatory effect of Ex-phe1 far outweighed that of Ex4, whilst also reducing the degree of nausea assessed in a behavioural study. Here, Ex-phe1 caused greater insulin secretion (unlike P5). This also translated into greater glucoregulatory abilities in a chronic study, and improved resolution of hepatic steatosis, further highlighting the potential for biased GLP-1R agonists for improving GLP-1R therapies. N-terminal amino acid modifications are also a feature of a recently described acylated G protein-

biased agonist, which showed similar improvements in chronic glucose tolerance *versus* semaglutide and, interestingly, greater weight loss[504]. Altering the amino acids in GLP-1 from the canonical α-form to the β form similarly results in a β-arrestin biased GLP-1R agonist, through altered extracellular interactions with the receptor[505].

Recently, a previously discovered small-molecule agonist at the GLP-1R (TT-OAD2) was discovered to be cAMP-biased compared to GLP-1[506]. Discovery of small molecule ligands at the GLP-1R is in its infancy, due to the difficulty of overcoming the "two-state" activation model which dictates that both the orthosteric, transmembrane binding pocket and allosteric extracellular domain must be bound before the receptor is activated[507,508]. It is now appreciated that GLP-1R activation is more complex than this, with intrinsic signal bias, spatiotemporal signalling and heterodimerisation affect GLP-1R activation and signalling[509]. However, small molecules may be able to activate the GLP-1 in a non-orthosteric function to elicit bias[510]. Regardless, TT-OAD2 binds uniquely within the transmembrane domain to elicit cAMP-biased signalling, expanding the inventory of ligands available to selectively activate the GLP-1R[506].

In addition to the ability of orthosteric ligands to confer unique receptor conformations which induce selective signalling of intracellular pathways at the GLP-1R, it has also been shown that positive allosteric modulators (PAMs) can affect signalling at the GLP-1R. For example, Koole *et al.* showed that the Novo Nordisk compound "compound 2" selectively enhanced OXM-mediated cAMP signalling, with negligible effects with GLP-1(7-36NH_2) or Ex4[511]. In the same study a second PAM, quercetin, selectively enhanced GLP-1(7-36NH_2) and Ex4-mediated Ca^{2+} signalling with little effect on OXM signalling. Similarly, *trans*-isomer derivatives of the GLP-1R PAM 4-(3-(benzyloxy)phenyl)-2-(ethylsulfinyl)-6-(trifluoro-methyl)pyramidine (BETP) enhanced cAMP signalling at the GLP-1R in response to GLP-1(9-36NH_2) and GLP-1(7-36NH_2) without significant effects on β-arrestin recruitment[512]. Therefore, it is evident that signal bias can be controlled by both orthosteric ligands and allosteric modulators.

There are currently no studies which explicitly investigate the therapeutic potential of biased signalling at the GCGR. This is striking as activation of the glucagon receptor is associated with energy expenditure and weight loss[227,228,382] and contributes to insulin secretion[12,391,513], yet has the disadvantageous effect (from the point of view of

a candidate for diabetes treatment) of augmenting gluconeogenesis[358]. Therefore, deeper understanding and delineation of the pathways associated with GCGR signalling could yield agonists which engage in pathways which activate energy expenditure and/or insulin secretion, whilst limiting hepatic gluconeogenesis, thereby producing a safer way to achieve the beneficial effects of GCGR agonism in T2DM. $G\alpha_q$ and β-arrestins functionally bind to the GCGR to induce desensitisation and receptor internalisation[283,403], and $G\alpha_q$ may also be implicated in gluconeogenesis and ureagenesis[399]. Intelligent design would therefore minimise $G\alpha_q$ signalling to reduce amino acid flux and hyperglycaemia, and by bypassing β-arrestin recruitment there could be a prolongation of signalling which would increase energy expenditure and insulin secretion. Whilst prolonged signalling could increase hyperglycaemia through Gαs-mediated pathways, concomitant insulinotropic GLP-1R activation would counteract this. Similarly, allosteric modulation of GCGR activity by endogenous proteins such as receptor activity modifying protein 2 (RAMP2), has been described, raising the possibility that synthetic PAMs could be used to selectively modulate GCGR signalling, as has been achieved for the GLP-1R using “compound 2”, quercetin and *trans*-BETP[511,512].

Finally, an example of a biased dual GLP-1R/GIPR agonist, P18, has recently been described by Al-Zamel *et al*[514]. P18, a peptide first developed by Finan et al. in 2013[456], was equipotent to GLP-1 at the GLP-1R for cAMP and more potent than GIP at the GIPR. However, P18 displayed reduced efficacy for β-arrestin 2 recruitment than GLP-1 at its receptor, whereas the β-arrestin recruitment response was not measured at the GIPR. This provides the first-in-class biased dual incretin agonist, however much greater work needs to be performed before understanding how this bias affects the agonist physiology. One would predict, however, that it would increase insulin secretion and improve glycaemic control, as Gαs-biased GLP-1R mono-agonists are superior to their unbiased comparators[276,502,504] and this likely translates when additional receptor activation is added. It becomes increasingly more complex to conceive the idea of biased dual agonists, as there are two receptors, for which the relative potency of the ligand to either receptor can be different, as well as multiple intracellular pathways. However, by utilising previous knowledge produced on receptor signalling and previously published biased agonists, more accurate

predictions can be made when designing and testing biased dual agonists *in vitro*, and how that could translate to improved therapeutic value *in vivo*.

1.6 Conclusion & Aims

T2DM is a serious threat to global health and is closely associated to obesity (termed "diabesity") and modern lifestyles. Current pharmacotherapies are not sufficient at reducing both the hyperglycaemia and excessive weight associated with diabesity. Therefore, improvements in this field are vital to public health and the global health economy. Drugs mimicking the actions of OXM show clear improvements in therapeutic potential compared to GLP-1R agonists alone, resulting in a greater weight loss through GCGR signalling and improved anti-hyperglycaemic profile via GLP-1R mechanisms. However, like GLP-1R agonists, dual GLP-1R/GCGR agonists are not without their limitations, namely that they induce nausea amongst other side effects which limits tolerability. Therefore, doses administered are sub-optimal, meaning there is still scope to improve dual agonist therapies. G protein-biased GLP-1R have shown improved tolerability as well as an improvement in chronic anti-hyperglycaemia by reducing receptor internalisation. Therefore, it is possible that similar mechanisms could be applied to dual GLP-1R/GCGR agonists. This has not yet been investigated, however the integration of G protein bias into dual GLP-1R/GCGR agonists may allow for a more tolerable and efficacious anti-diabetes and anti-obesity drug. The potential impact of this study is great and could expand the exciting potential that biased signalling now allows for drugs targeting GPCRs.

Therefore, the aims of this project are:

1) Identify examples of dual GLP-1R/GCGR agonists which display signalling bias toward G protein/cAMP at either the GLP-1R or GCGR.
2) Investigate how G protein bias with dual agonists affects chronic signalling *in vitro.*
3) Examine the effects of G protein-bias *in vivo* using lean and diet-induced obese (DIO) mice to investigate the therapeutic potential of G protein-biased dual agonists.

2 Methods

2.1 Peptides

GLP-1(7-36)NH_2 and GCG were obtained from Bachem (Switzerland). Custom peptides were synthesised by Insight Biotechnology (UK) and WuXi AppTec Biologics (China). Sequences are shown in the Appendix.

2.2. Animals

All animals used were housed in a 12-hour light/dark cycle room. Wild-type male C57BL/6 mice were provided by Envigo (3 months – 1 year old) and were fed on either a standard chow diet (RM1(E), Special Diets Services, UK) or 60% high-fat diet (HFD) chow (D12492, Research Diets Inc., UK) as stated in the text, with water available *ad libitum*. Transgenic mice were provided by the collaborators as listed. Rats were provided by Charles River (250-500g) and were fed on standard RM1(E) chow diet with water provided *ad libitum*. Mice were housed either individually or grouped house as 4 per cage. Rats were individually caged for all experiments. For all grouped studies, animals were randomly allocated into weight-matched groups. Peptide administration sequence was also randomised to prevent treatment sequence effect. All procedures were carried out in accordance with the Animal (Scientific Procedures) Act 1986.

2.3 Maintenance of Transgenic Mouse Lines

Transgenic mice were generated to confirm the role of GCGR signalling in biased dual GLP-1R/GCGR agonist pharmacology. Dr Victoria Salem (Imperial College London) kindly provided C57BL/6J mice containing LoxP sites flanking the *Gcgr* gene (*Gcgr*$^{fl/fl}$). *Gcgr*$^{fl/fl}$ mice were crossed with C57BL/6J mice containing Cre-recombinase under the control of the *Albumin* promoter (*Alb-Cre*) to create hepatocyte-specific *Gcgr* knockdown mice (*Alb-Cre:Gcgr*$^{fl/fl}$ or *Gcgr*$^{hep-/-}$), or Cre-recombinase under the control of the β actin promoter fused to a modified oestrogen receptor sequence (*Actb-Cre-ERT2*) to create a tamoxifen-inducible global Gcgr knockdown line (*Actb-Cre-ERT2:Gcgr*$^{fl/fl}$ or *Gcgr*$^{-/-}$) (both Cre lines kindly provided by Prof David Carling, Imperial College London). To induce global knockout with tamoxifen, mice were orally gavaged with

100mg/kg tamoxifen in corn oil (Thermo Fisher, USA) for 5 consecutive days followed by a 7-day recovery period.

2.4 Transgenic Strain Genotyping

Genotyping was performed with a tissue sample acquired from each individual animal using the KAPA2G Fast HotStart Genotyping system (KAPA Biosystems, USA). Here, DNA was extracted from the tissue using the KAPA Extraction enzyme system using the following protocol:

Duration	**Temperature**
10min	75°C
5min	90°C

The extracted DNA was maintained in 10mM Tris HCl buffer (pH 8.5) at -20°C.

Appearance of *Gcgr*$^{fl/fl}$ and *Alb-Cre* or *Actb-Cre-ERT2* were identified using the polymerase chain reaction (PCR), with the following primers (Sigma, USA):

Gcgr forward – 5'- GGGCCAGCTCTGATTGTGT -3'
Gcgr reverse – 5'- GAAGGGCCATGGTAGGACA -3'
Cre forward – 5'- CGTACTGACGGTGGGAGAAT-3'
Cre reverse – 5'- CCCGGCAAAACAGGTAGTTA-3'

Here, the *Cre* primers were based on a generic Cre primer sequence. Primer sequences were kindly provided by Dr Phillip Muckett and Mr Yateen Patel (Imperial College London).

PCR amplification was performed using the following protocol (35 cycles):

Duration	**Temperature**	
3min	95°C	
15sec	95°C	35 cycles
15sec	60°C	
15sec	72°C	
1min	72°C	

The corresponding PCR product was run on a 2% agarose gel with 0.01% (v/v) ethidium bromide, and imaged using the Syngene G:BOX Chemi XRQ gel doc system (UK) with the GeneSys image acquisition software (version 1.0.1).

2.5 Cell Culture

Cell lines used were: PathHunter® Chinese hamster ovary (CHO)-K1 human (h)GLP-1R-β-arrestin-2, CHO-K1-hGCGR-β-arrestin-1 and -β-arrestin-2 (DiscoverX, USA), Huh7 hepatoma cells stably expressing GCGR, INS1-832/3 rat β cell-like cell line and Human Embryonic Kidney-293 cells optimised for transfection (HEK293T) cells. Cells were maintained at 37°C perfused with 95%:5% ratio CO_2. PathHunter® CHO-K1 cells were maintained in Ham's F-12 Nutrient Mixture (Gibco, USA) supplemented with 1mM L-glutamine, 10% foetal bovine serum (FBS) (Thermo Fisher, USA), 5mg/l penicillin/streptomycin (Sigma, USA), 1mg/l G418 and 0.4mg/l hygromycin. Huh7 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) containing 10% FBS, 1% penicillin/streptomycin and 1mg/l G418. INS-1 832/3 mouse β cell lines were a kind gift from Prof Christopher Newgard, Duke University) and Dr David Hornigold (MedImmune, UK) and were maintained in Rosa Park Memorial Institute (RPMI)-1640 media (Sigma, USA) containing 11mM glucose, 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2mM glutamine, 1mM sodium pyruvate, 50μM β-mercaptoethanol, 10% FBS and 1% penicillin/streptomycin. HEK293T cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin, and transfected in serum-free media without antibiotics present.

2.6 Cyclic AMP Assay

cAMP accumulation was measured in stimulated PathHunter® CHO-K1 cells, Huh7 cells over-expressing hGCGR, HEK293T cells transiently transfected with either SNAP-tagged GLP-1R or GCGR, or INS-1 832/3 cells. Analysis of cAMP was confirmed using homogenous time resolved fluorescence (HTRF), specifically the cAMP Gs Dynamic 2 cAMP kit (Cisbio, France). A number of variations on the protocol were used. In all assays, cells were plated with 10,000 cells per well of a 96-well plate. CHO-K1 cells were assayed in suspension, and therefore treated immediately with peptide. Huh7 and INS-1 832/3 cells were plated at approximately 5,000 cells per well

the night before the assay and treated with peptide the following day. HEK293T cells were plated at approximately 50% confluency in a 12 well plate and transfected overnight the following day with 1µg receptor in Lipofectamine 2000. Transfected cells were lifted and assayed the day after in a separate white 96-well plate. Plated cells were incubated with the respective peptide for either 30 minutes (acute incubation), 8 hours or 16 hours (chronic incubation, as per figure legend) at 37°C. Levels of cAMP were analysed as per manufacturer's instructions. Results were expressed relative either to the endogenous ligand (i.e. glucagon for the GCGR cells), to the parent compound or to 10µM forskolin (as indicated in the respective figure legend). No phosphodiesterase inhibitor was used, except for transfected HEK293T cells and INS-1 832/3 cells, in which 500µM 3-isobutyl-1-methylxanthine (IBMX) was added for the final 15 minutes of the stimulation period before lysis. Fluorescence was measured using the Spectramax® i3x Multi-Mode Detection Platform and read using SoftMax Pro version 6.5.1 (both Molecular Devices, USA).

2.7 PathHunter® β-Arrestin Assay

β-arrestin recruitment was determined in PathHunter® CHO-K1 hGLP-1R and hGCGR βArr2 cells using the PathHunter® assay kit (DiscoverX). Here, cells were plated at 10,00 cells per well and immediately stimulated with peptide for 30 minutes at 37°C. Detection reagent was made up and applied as per manufacturer's instructions. Results were expressed relative either to the endogenous ligand or the parent compound (as indicated in the respective figure legend). Samples were read as for cAMP assays.

2.8 Receptor Internalisation Measurement by Wide Field Microscopy

Approximately 500,000 HEK293T cells were plated onto 13mm circular glass coverslips (Agar Scientific, UK) in a 24-well plate, and were then transfected with SNAP-hGLP-1R or SNAP-hGCGR using Lipofectamine 2000 transfection reagent (Sigma, USA). The next day, cells were labelled using 500nM SNAP-Surface-549 (New England Biolabs) in complete DMEM for 30 minutes at 37°C to label the receptor before washing, followed by treatment with 1µM peptide in serum free DMEM + 0.1% BSA for 30 minutes or 4 hours. Cells were then fixed with 4% PFA and mounted in Diamond Prolong mounting medium with 4',6-diamidino-2-phenylindole (DAPI)

(Invitrogen, USA). Slides were analysed using a widefield fluorescence microscope (Nikon Eclipse Ti2) controlled by μManager. Cells were selected at random from across the slide as representative of the population and three to four images taken per slide using the TRITC and FITC filter sets. Images obtained were analysed and manipulated using Fiji[515] on ImageJ (version 1.52, USA)[516].

2.9 Gα & βArr2 NanoBit Recruitment Assay

The complimentary NanoBit luciferase was formed on two subunits: the small subunit (SmBit; to be attached to the receptor) and large subunit (LgBit; attached to the signal transducer). The plasmid for MiniGs (a kind gift from Prof Nevin Lambert, Medical School of Georgia[517]) as well as the plasmid containing β-arrestin-2 (Promega plasmid # CS1603B118[518]) all contained the LgBit on the N-terminal on arrival. The plasmids FLAG GLP-1R-Tango and FLAG GCGR-Tango were kindly provided by Dr Bryan Roth (Addgene #66295)[519].

Firstly, the SmBit oligomer (Sigma, USA) was annealed using the following method:

SmBit sequence:
5'- gtgaccggctaccggctgttcgaggagattctgtaa - 3'
3' – cactggccgatggccgacaagctcctctaagacatt - 5'

- 10μM of each oligomer
- 5μl annealing buffer (100mM Tris, 10mM EDTA, 500mM NaCl in MilliQ water)
- Up to 50μl total reaction volume with RNAse-free water

Duration	**Temperature**
4min	95°C
10min	70°C
overnight	25°C

The TANGO cassette was excised and replaced with the SmBit oligonucleotide in the FLAG-GLP-1R-TANGO and FLAG-GCGR-TANGO plasmids using AgeI-HF and XbaI

restriction enzymes, and the SmBit ligated into the plasmid at a 10:1 molar ratio using T4 DNA ligase (1:10 volume ratio; Promega, USA) at 4°C overnight. Successfully ligated plasmid was isolated using gel electrophoresis with a 1% agarose gel containing 1:50,000 SYBR Safe DNA Gel stain (Thermo Fisher, USA) and extracted using the DNA Gel Extraction kit (New England Biolabs, USA).

HEK293T cells were plated the day before transfection to reach ~70% confluency on the day of transfection. Cells were consequently transfected for 24 hours in Lipofectamine 2000 with 0.5µg of the GLP-1R-SmBit or GCGR-SmBit plasmid along with 0.5µg of either MiniG-LgBit or β-arrestin-2-LgBit. Cells were resuspended in Nano-Glo dilution buffer with fumarizine (Promega, USA) and seeded in 96-well plates. Basal luminescence was measured for 5 minutes using the FlexStation 3 plate reader (Molecular Devices, USA) at 37°C before addition of peptide or vehicle (0.9% NaCl saline). Luminescent signal was subsequently monitored for 30 minutes, with responses normalised to the average baseline luminescence. Data was analysed using area under the curve (AUC) analysis.

2.10 Quantitative PCR (qPCR)

Sections of liver and kidney were taken from *Gcgr*$^{-/-}$, *Gcgr*$^{hep-/-}$ and littermate control mice for confirmation of knockdown. Samples were extracted from culled animals, flash frozen in liquid nitrogen and stored at -80°C. Frozen samples (~10mm^3) of each tissue were homogenised using the TissueLyser II (Qiagen, Germany) and RNA extracted using a modified version of the PureLink™ RNA Mini Kit (Thermo Fisher, USA). Here, in between washing steps, extracted RNA was incubated for 15 minutes with DNAse (Thermo Fisher, USA) to degrade any remaining DNA in the system.

500ng of extracted RNA was used to synthesise cDNA using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher, USA), following manufacturer's instructions.

GCGR expression was quantified in the corresponding cDNA using TaqMan Gene Expression Mastermix (Thermo Fisher, USA) with a validated TaqMan GCGR probe (Mm01248725_g1, Thermo Fisher, USA) which targets exon 7 to 8 of the *Gcgr*, and

cyclophilin a (Mm00510343_m1) as the housekeeping gene. Data is presented as a percentage knockdown relative to the littermate control *Gcgr* expression.

2.11 Acute Intraperitoneal Glucose Tolerance Test

Lean or HFD mice were fasted for at least 4 hours before commencing the glucose tolerance test, depending on the peptide treatment length. Mice were injected into the intraperitoneal (i.p.) cavity with peptide (dose described in figure legend) or vehicle either 8-hours before, 4-hours before or at the same as the glucose challenge (acute). Glucose was dosed at 2g/kg body weight. Blood glucose levels were measured before glucose challenge, then at the times as indicated in the figure using the GlucoRx® Nexus glucose meter (GlucoRx, UK).

2.12 Intraperitoneal Insulin Tolerance Test

Lean mice were fasted for 2 hours before i.p. injection of 10nmol/kg SRB103Gln, SRB103His or vehicle. 4 hours later, baseline blood glucose was taken before insulin (Sigma, USA) (0.5U/kg – 1U/kg) was injected i.p. and blood glucose measured 20, 40 and 60 minutes after insulin injection using the GlucoRx® Nexus glucose meter (GlucoRx, UK).

2.13 In vivo Insulin Assay

Lean C57BL/6 mice were fasted for 4 hours before i.p. injection of peptide (10nmol/kg) or vehicle. Plasma insulin and glucose levels were subsequently measured 30 minutes, 4 hours and 8 hours post peptide injection. Here, venesections were performed both before and 10 minutes after a 2g/kg i.p. glucose injection to measure the insulin response. Insulin was measured by isolating plasma by centrifuging at 10,000 g for 8 minutes at 4°C using the Sigma 3-16KL refrigerated centrifuge (Sigma, USA) and read using the Cisbio Insulin mouse serum assay kit (Cisbio, France).

2.14 Acute Feeding Study

Individually caged C57BL/6 mice (both lean and DIO) were fasted 16 hours before the start of the study. On the day of the study, mice were i.p. injected with vehicle or peptide (10nmol/kg). Thirty minutes after injection, food was reintroduced to the mice

and food weight measured 1 hour, 2 hours, 4 hours and 8 hours post injection. Food intake was calculated relative to the weight of food reintroduced to each animal.

2.15 Chronic Feeding Study

SRB103 peptides were made up in zinc chloride ($ZnCl_2$) solution, made up in water for injection (WFI), to a molar ratio of 1.2:1 ($ZnCl_2$:peptide) and liraglutide was made up in WFI. DIO mice (approximately 7-9 months, mean weight 43-46g) received daily subcutaneous (s.c.) injections of the treatments (n=9-10) mentioned. For the low-dose study, the following dose-titration occurred: day 1-3: 10nmol/kg, day 4-6: 16.7nmol/kg, day 7-15: 20nmol/kg. For the high-dose study, the following dose titration occurred: day 1-3: 16nmol/kg, day 4-6: 32nmol/kg, day 7-21: 50nmol/kg. Body weight and food intake was measured periodically, with food and water available *ad libitum*.

2.16 Chronic Intraperitoneal Glucose Tolerance Test

On the day of the study, mice were fasted in the morning an hour before s.c. injection as stated. 8 hours later, baseline blood glucose was measured before glucose (2g/kg) was injected i.p. and subsequently 20, 40, 60 and 90 minutes after glucose injection (as stated in the figure) using the GlucoRx® Nexus glucose meter (GlucoRx, UK).

2.17 Body Composition Analysis

Body composition was assessed in live DIO mice on day 0, day 13 (the day before the IPGTT) and day 21 (end of study) of the high-dose chronic study and day 0 and day 16 (day after IPGTT and end of study) of the low dose chronic study using magnetic resonance imaging (EchoMRI-100, Echo Medical System, USA). Fat mass, lean mass, free water and total water were measured and expressed as a percentage relative to day 0 results.

2.18 DPP-IV Degradation Study

10mM of SRB103Gln, SRB103His or GLP-1 was dissolved in buffer (100mM Tris-HCl, pH8), and treated with either 7μM mouse recombinant DPP-IV (R&D Systems, USA) or vehicle (n=3). Immediately, 1 hour after or 24 hours after DPP-IV treatment, the reaction was terminated by adding TFA to a final concentration of 0.1% and the

peptide sample read using the 1200 Series high performance liquid chromatography (HPLC) machine (Agilent, USA) with the Aeris Peptide 3.6μm XB-C18 column (Phenomenex, USA). Degradation was measured as the percentage degradation from the 0h sample.

2.19 Pharmacokinetic Study

Ad libitum fed DIO mice were administered 0.5mg/kg peptide via i.p. injection. 4 hours after injection, blood was acquired by venesection into lithium heparin-coated microvette tubes (Sarstedt, Germany). Plasma was separated by centrifugation at 10,000 g for 8 minutes at 4°C. Plasma concentrations were assessed by radioimmunoassay using an in-house designed radiolabelled antibody selective for peptides within the provided peptide library.

2.20 Bias Quantification

Signalling bias was calculated using three methods. Firstly, a modified version of the operational model of pharmacological agonism was used[476]. Concentration response data were fit to the equation below to derive the transduction ratio (τ/K_A) for all agonists for either cAMP production or β-arrestin recruitment:

$$Response = basal + \frac{(\mathrm{Emax} - basal)(\frac{\tau}{K_A})^n[A]^n}{[A]^n(\frac{\tau}{K_A})^n + (1 + \frac{[A]}{K_A}^n)}$$

Where:

basal – baseline response of the assay

E_{max} – maximum response of the assay

τ – efficacy of agonist to the pathway

K_A – affinity of the agonist to the pathway

A – agonist concentration (M)

n – “transducer slope” linking agonist concentration to its response

Secondly, the relative activity scale ($\Delta\log(E_{max}/EC_{50})$ was used. Both E_{max} and EC_{50} were calculated from the 4-parameter fit curves for cAMP production and β-arrestin-2 recruitment on an assay-by-assay basis.

In all cases, baseline and E_{max} were constrained to fit globally, as they are assay specific parameters. Bias for SRB103Gln3 at either pathway was calculated by normalising $\log(\tau/K_A)$ or $\log(EC_{50}/E_{max})$ for the agonist in question to the reference cognate ligand (either glucagon or GLP-1) to create a $\Delta\log(\tau/K_A)$ or $\Delta\log(EC_{50}/E_{max})$ value. Bias between the two pathways for the agonists was calculated by subtracting the $\Delta\log(\tau/K_A)$ or $\Delta\log(EC_{50}/E_{max})$ value generated for β-arrestin-2 recruitment from that generated for cAMP recruitment. This is example for the modified operational model at the GLP-1R is shown below:

Pathway-specific bias:

GLP-1R β-arrestin-2: $\Delta\log(\tau/K_A)_{[\beta\text{-arrestin-2}]} = \Delta\log(\tau/K_A)_{[agonist]} - \Delta\log(\tau/K_A)_{[GLP\text{-}1]}$

GLP-1R cAMP: $\Delta\log(\tau/K_A)_{[cAMP]} = \Delta\log(\tau/K_A)_{[agonist]} - \Delta\log(\tau/K_A)_{[GLP\text{-}1]}$

Bias between pathways at the GLP-1R:

$\Delta\Delta\log(\tau/K_A)agonist = \Delta\log(\tau/K_A)_{[cAMP]} - \Delta\log(\tau/K_A)_{[\beta\text{-arrestin-2}]}$

This structure also applies for the relative activity scale.

The “competitive analysis” model was used to better define the transduction ratio (τ/K_A) in cases of very partial agonists, where the modified operational model was not sufficient[479]. Here, data was fitted to the following equation:

$$Response = basal + \frac{(Emax - basal)}{1 + (\frac{1 + 10^{(A+LogK_{reference})} + 10^{(X+LogK_{Test})}}{10^{(A+LogR)} + 10^{(X+LogR+LogRA)}})^{n}}$$

Where:

basal – baseline response of the assay

E_{max} – maximum response of the assay

A – log concentration of the reference agonist (M)

LogK – log affinity constant

X – log concentration of the test agonist (M)

LogR – log transduction coefficient (τ/K_A)

LogRA – the difference in LogR between the test and reference compound

2.21 Statistical Analysis

All data were analysed using GraphPad Prism version 7.00 or 8.00 (GraphPad Software, USA). For all dose-response curves, data was fitted with a 4-parameter curve, apart from biased analysis using the modified operational model and "competitive analysis", where curves were fitted to the appropriate equation (see section 2.20). Student's t-test was used for the analysis of bias to analysis statistical difference between two treatments, one-way ANOVA with Tukey post hoc test was used to determine statistically significant differences between three or more treatments, two-way ANOVA with Tukey post hoc test to compared time and treatment, and three-way ANOVA with Tukey post hoc test to compared time, treatment and genotype for all treatment groups. All data presented is expressed as mean ± standard error of mean (SEM) unless stated otherwise, with individual values displayed where appropriate.

3 In vitro identification of biased dual GLP-1R/GCGR agonists

3.1 Introduction

The discovery and application of signalling bias with GLP-1RAs has been identified as a promising therapeutic approach to improve their use for treating obesity and diabetes[276,502,504,520]. In this chapter established and novel techniques are applied to identify and pharmacologically evaluate biased signalling of dual GLP-1R/GCGR agonists, a promising emerging treatment for metabolic disorders.

3.1.1 Biased GLP-1R agonists

A number of biased GLP-1R agonists have been described which show distinct patterns of intracellular signalling, with variable coupling to different pathways, including cAMP, ERK1/2 phosphorylation and Ca^{2+} accumulation. As the N-terminus of GLP-1 has been shown to be critical in the activation of GLP-1R[521-524], *Jones et al.* developed a panel of Ex4-derived GLP-1R agonists with N-terminal substitutions which showed signalling bias toward cAMP signalling or β-arrestin recruitment[276]. For example, the cAMP-biased Ex4 peptide, Ex-phe1, showed reduced receptor dissociation kinetics and lower potency for acute cAMP production than Ex4, but produced greater insulin secretion upon chronic stimulation due to markedly reduced β-arrestin-mediated receptor internalisation. Conversely, a β-arrestin-biased Ex4 analogue, Ex-asp3, showed a rapid loss of GLP-1R signalling upon chronic stimulation due to increased receptor internalisation. Functionally, Ex-phe1 showed greater anti-hyperglycaemic performance during an intraperitoneal glucose tolerance test (IPGTT) over longer treatment periods compared to Ex4, despite identical pharmacokinetics. However, there were no changes to food intake and an improvement of pica, a rodent model of nausea, suggesting a greater drug tolerability to Ex-phe1 compared to Ex4.

Exendin-P5 (P5) is another example of a modified Ex4-based peptide showing distinctive intracellular signalling patterns[502]. P5 showed potency for cAMP production similar to that of GLP-1, however slightly less than Ex4. P5 also showed three-fold greater Gαq-mediated Ca^{2+} mobilisation than did Ex4, yet reduced efficacy and potency for both β-arrestin-1 and -2 recruitment. This resulted in reduced receptor desensitisation and improved anti-hyperglycaemia chronically compared to Ex4 in diabetic *ob/ob* mice (discussed in section 4.1.2). This was also accompanied by improvements in metabolic profile. Recently, this group developed an acylated G-

protein biased GLP-1R agonist based on the structure of Ex4, in a similar method used to generate P5[504]. Here, “P17” showed improved glucoregulatory effects, both acutely and chronically, as well as improved hepatic steatosis and decreased weight gain and food intake compared to semaglutide.

Another “biased” GLP-1R agonist recently published was by Zhong *et al.*, where they describe an extracellular domain-biased agonist[520]. Here, a 12 amino acid peptide displaying high affinity to the extracellular domain was attached to Ex4, called PEP20. PEP20 showed an improved acute anti-hyperglycaemic effect than liraglutide in DIO mice, however in chronic studies PEP20 displayed similar effects to liraglutide for reducing body weight and food intake, but surprisingly, a slightly worse glucose tolerance than liraglutide. PEP20 was also not tested for its affinity to the orthosteric binding site, therefore it is unknown whether it truly is “biased” toward the extracellular domain. However, it provides another method of designing biased ligands through targeting a particular binding site as opposed to recruitment of a particular signal transducer.

Substitution of α-amino acids to β-amino acids at selected site on the GLP-1 peptide also results in GLP-1R agonists with varying degrees of signalling bias, from cAMP-selective to β-arrestin-selective[505,525]. The functional effect of this is yet to be described but allows for further insight into β-arrestin-mediated biology at the GLP-1R.

3.1.2 The rationale for biased GCGR agonism in metabolic disease

Glucagon receptor activation is linked to a number of metabolic effects. Traditionally, it was associated with anti-hypoglycaemia through activation of hepatic glycogenolysis and gluconeogenesis to increase glucose output[2]. In cases of hypoglycaemia, such as insulin overdose, insulinomas and non-insulinoma pancreatogenic hypoglycaemia syndrome (NIPHS), glucagon can be used therapeutically to increase circulating glucose levels [526-528]. For GCGR-based therapies to be used to treat type 2 diabetes, this hyperglycaemic effect is clearly detrimental as this would exacerbate the chronic hyperglycaemia already present in type 2 diabetes.

Glucagon is also implicated in amino acid catabolism, in an indirect manner. As a driver of gluconeogenesis where glucose is synthesised from non-carbohydrate sources such as amino acids, administration of glucagon results in hypoaminoacidaemia and enhanced protein catabolism[358-360,529,530]. In glucagonomas, which result in chronic elevation of glucagon, one of the hallmark signs is a reduction in lean muscle mass[351]. As skeletal muscle is an intrinsic tissue for storing excess circulating glucose[531,532], it again stands that this physiological side effect of glucagon administration makes it unattractive as a therapeutic strategy for patients with type 2 diabetes.

Whilst current therapeutic uses of glucagon are centred around correction of hypoglycaemia, mounting evidence suggests its potential expands beyond this. Glucagon increases energy expenditure and induce weight loss in rodents[380,533,534], dogs[535], pigs[536] and humans[228,381,537]. The pathways associated with glucagon-induced energy expenditure remain undefined and controversial, and numerous theories have been put forward BAT thermogenesis[383,385], amino acid catabolism[538,539] and centrally mediated energy expenditure[534,540]. With further clarification on the molecular mechanisms involved in GCGR-mediated energy expenditure, it would be interesting to see how bias can be utilised to harness the energy expenditure abilities of glucagon without the side effects of chronic GCGR stimulation such as amino acid catabolism.

The understanding of GCGR pharmacology is also increasing, which will improve the design of GCGR-based therapies. Like GLP-1R, GCGR internalisation is mediated by β-arrestin recruitment, however, in hepatocytes β-arrestin-2 appears to be the isoform responsible for GCGR internalisation[283]. Mice lacking hepatic-specific β-arrestin-2 but not β-arrestin1 showed reduced acute glucose tolerance and pyruvate tolerance, a result of enhanced GCGR signalling. β-arrestin-1 knockout mice were metabolically normal, suggesting divergent effects of the β-arrestins in the liver. Kim *et al.* described recently how mice co-treated with insulin and a GCGR agonist paradoxically showed decreased endogenous glucose production and improved insulin sensitivity, as evidenced by increased glucose infusion rates in hyperinsulinaemic clamp studies and increased insulin receptor phosphorylation[394]. As GCGR is present on the pancreatic β cell[541], and glucagon acts as an agonist at the GCGR and GLP-1R on the β cell[12,513],

it is suggested that glucagon plays an important role in regulating insulin secretion which can be harnessed with the dual agonists.

As a treatment for obese diabetics, physiological effects of energy expenditure and increased insulin sensitivity should be exploited to improve weight loss and diabetic management. However, glucagon-based therapies are hampered by glucagon-induced hyperglycaemia and amino acid catabolism. Expanding our understanding of how the GCGR signals to initiate all of its effects will hopefully lead to the design of agonists which can exploit the positive metabolic effects of GCGR agonists and mitigate major hyperglycaemia and muscle wastage which hamper its use as a metabolic therapy target. Evaluating the effects of glucagon described in this section, it can be hypothesised that a glucagon receptor agonist with low acute receptor potency, hence producing a reduced acute hyperglycaemic response, but prolonged signalling abilities, such that insulin sensitivity and energy expenditure are maintained and enhanced may provide a therapeutically viable GCGR agonist.

3.1.3 Measurement of protein-protein interaction and G protein recruitment

Whilst other pathways have been shown to be involved in GLP-1R-mediated insulin release[542,543], it is generally accepted that the Gαs-AC-cAMP pathway is the most critical step and likely regulates some of the other described pathways such as Ca^{2+} mobilisation and ERK1/2 phosphorylation[542,544]. Much less is known of the biology of GCGR; however, currently it is agreed that cAMP is associated with the canonical effects of glucagon including glucose, lipid and amino acid regulation[397,545-547]. Measuring activation of the $G\alpha_s$-AC-cAMP signalling pathway is therefore a good measure in both receptors to measure efficacy of the drug.

There are a great variety of ways to explore this pathway. Firstly, the dissociation of Gα from the G protein heterotrimer, a measure of G protein activation, can be measured. One of the first ways developed to measure this was the [^{35}S]GTPγS binding assay. This uses a labelled, non-hydrolysable form of ATP, [^{35}S]GTPγS, to measure the recruitment of GTP to the Gα subunit, and thus initiation of the heterotrimer dissociation[548]. Whilst this assay is relatively simple to set up and

produces good detectable responses, this approach uses the radiolabel [^{35}S] so studies must be terminal, and responses to $G\alpha_s$ are noticeably lower than for $G\alpha_i$[549]. Non-radioactive, europium-labelled GTPγS is available however binding affinity is ten-fold lower than [^{35}S]-labelled[550]. Kinetics of G protein dissociation, including for $G\alpha_s$, can be measured using a standard bioluminescence resonance energy transfer (BRET)-based assays[551] or a recently developed nanoBRET system[552]. Whilst standard BRET systems use relatively large luciferases (typically the 61kDa Firefly or 36kDa Renilla luciferase) to transfer energy to a fluorescent acceptor molecule, nanoBRET system uses the smaller (19kDa) NanoLuc luciferase[553]. This smaller NanoLuc luciferase reduces steric inhibition of protein-protein interactions in the cell, improving the validity of BRET-based assays. Another approaches to investigate protein-protein interactions is Förster resonance energy transfer (FRET), which uses an excitation source to initiate energy transfer from the donor to the acceptor. However, due to the need for an external excitation source, FRET can cause photobleaching of samples, autofluorescence and adverse stimulation of additional biological processes[554]. BRET technologies can, however, lack signal strength compared to FRET which can cause issues when using low sensitivity equipment. The relatively low number of BRET donors can also limit the scope of its use.

Another readout of this pathway is the measurement of Gα recruitment to the receptor. The main benefit of measuring Gα recruitment over measuring G protein heterotrimer dissociation is that this is a direct measure of the initiation of the pathway of interest. BRET and NanoBRET have been extensively used to measure Gα recruitment[555,556]. NanoLuc can also be used in a protein-fragment complementation assay (PCA), called NanoBiT[557]. Whilst other PCAs are available to measure protein-protein interactions, the large size and high affinity of other fragmented luciferases makes them much less suitable for investigating transient or weaker intracellular protein-protein interactions[557]. The two subunits have low affinity and therefore won't bind under non-physiological conditions, and association occurs when the two portions are in close proximity[557]. The NanoBiT system also gives a greater signal to background ratio compared to NanoBRET, meaning deviations in signals are easier to detect.

Looking further along the $G\alpha_s$ signalling cascade, many high-throughput screens (HTS) are available which measure cAMP accumulation. There are commercial high-

throughput assays, based on time-resolved Förster resonance energy transfer (TR-FRET) available to measure cAMP accumulation in cells, such as the HTRF Gs Dynamic Kit (Cisbio, France) and the Thunder™ TR-FRET kit (BioAuxilium, Canada). TR-FRET has advantages over regular FRET technology as the fluorophores used emit extended fluorescence which allows for a time delay between excitation of fluorophores and measurement of fluorescence, mitigating short-lived background noise. TR-FRET assays do however produce a low background to signal ratio, which can lead to variability in the data. Another HTS method for measuring cAMP is the HitHunter® cAMP assay (DiscoverX, USA), a PCA-based assay in which endogenous cAMP quenches antibodies allowing for light production from an activated luciferase. Other assays include enzyme-linked immunosorbent assay (ELISA) assays, which provide a greater sensitivity and accuracy of cAMP concentration which is useful for minute divergences in cAMP, however the protocol takes longer than the other HTS assays listed and are therefore not used in drug discovery. Radioimmunoassays (RIAs) can also be used to analyse cAMP production in vitro, however like the ELISA assays available, the time taken to perform this makes RIAs obsolete[558,559].

There is also great interest in the recruitment and biology of other Gα subtypes. As mentioned, $G\alpha_q$-mediated Ca^{2+} signalling is suggested to be important in an array of relevant physiology, including GLP-1R-mediated internalisation and insulin release[269] and GCGR-mediated glycogenolysis[400,560]. To measure Ca^{2+} flux *in vitro*, fluorescent dyes such as Fura-2 are used. Fura-2 remains popular since its introduction in 1985 due to its ability to provide quantitative data with minimal bleaching[561], however it only provides a macro-scale readout of the Ca^{2+} flux. Mechanistically, GCGR-coupled $G\alpha_q$ stimulates calcium-induced calcium release (CICR) from endoplasmic reticulum stores[400]. Measuring the biology and dynamics of CICR can be measured using BRET/FRET systems[562,563], or intracellular Ca^{2+} stores can be depleted using thapsigargin to knock out the ability for receptor-induced CICR, as has been explored with the GLP-1R[564-566] and GCGR[400]. Clearly, complete and sustained depletion of calcium stores using thapsigargin is not physiological, and causes mitochondrial permeability[567], which is detrimental to cells.

“RET” technologies have similarly been used to measure $G\alpha_i$ activation and recruitment to different receptors[568]. $G\alpha_i$ is of interest in the field of diabetes and

obesity as Gαi has specifically been implicated in both conditions[569,570] and $G\alpha_i$ recruitment has been observed at both GCGR[400] and GLP-1R[266,267]. Another method to investigate $G\alpha_i$ biology is using pertussis toxin (PTX). PTX catalyses the adenosine diphosphate (ADP)-ribosylation of $G\alpha_i$, locking the G protein subunit in its inactive, GDP-bound conformation and thus preventing interaction of $G\alpha_i$ with its receptor[571]. Treating with PTX *in vitro* is therefore a useful method of inhibiting $G\alpha_i$ and study the biology of $G\alpha_i$-interacting receptors such as GCGR and GLP-1R.

This work will primarily focus on $G\alpha_s$ biology and dynamics at both receptors for the reasons stated. Utilising the NanoBiT technology to investigate G protein recruitment kinetics will allow for an accurate representation of G protein recruitment to both the GLP-1R and GCGR, and provide the bright, discernible signal that can be difficult to get with nanoBRET assay. Accumulation of cAMP will also be shown using a commercial HTRF kit, which allows for accurate and quick quantification of the downstream activation events caused by $G\alpha_s$ recruitment and AC activation. In conjunction with each other, these two assays will provide detailed analysis of the effects caused by potential dual GLP-1R/GCGR agonists at the $G\alpha_s$-AC-cAMP pathway.

3.1.4 Measurement of β-arrestin recruitment

GPCR signalling is not only shaped by the pharmacological profile of the ligand or the kinetics of intracellular signalling pathways, such as the $G_{\alpha s}$-AC-cAMP pathway. The β-arrestin protein family plays a critical role in GPCR signalling kinetics, from prolongation to cessation of canonical and non-canonical intracellular signalling pathways[572-575]. New aspects of β-arrestin biology have also been discovered which provide evidence that β-arrestin can also act as a scaffold protein for distinct intracellular signalling pathways, including that of GLP-1R-mediated insulin release[275]. Therefore, measuring β-arrestin recruitment and activation upon ligand-receptor interaction is becoming equally important in drug discovery.

Many current methods for measuring β-arrestin recruitment use the same protein-protein interaction methodologies as discussed for G proteins including FRET[576-579], BRET[580-583], nanoBRET[584-586] and nanoBIT[557,587,588]. Like measuring G protein

activation and recruitment, these assays provide a real-time representation of β-arrestin recruitment but require system optimisation before reliable results can be obtained. A further assay is a luciferase-based assay, called TANGO-Presto, whereby luciferase mRNA is transcribed upon β-arrestin recruitment[589]. Whilst emitting high intensity signals, this assay does not provide real-time kinetic readouts of β-arrestin interaction and the C-terminus of the receptors used in this assay are modified to that of the V_2 vasopressin receptor (V_2R) to optimise β-arrestin recruitment, which effects validity of results obtained from this assay[519].

Due to increasing interest in β-arrestin recruitment and signalling, HTS assays also exist. The DiscoverX PathHunter® assay, which uses a luciferase PCA system to measure the recruitment of individual recombinant β-arrestin isoforms to a recombinant receptor, is one of the most commonly used[276,502,588,590-593]. The PathHunter® assay allows for rapid readouts of recruitment, with very little optimisation required. However, the recruitment of β-arrestin to receptor is not transient or reversible, unlike in normal physiology and therefore is not representative of the true physiology of β-arrestin recruitment.

Like Gα, methods exist which also measure β-arrestin activation. As β-arrestin activation is linked to conformational changes within the β-arrestin molecule, intramolecular FRET and BRET reporters exist which can measure the conformational change in the β-arrestin molecule, even after ligand-induced β-arrestin recruitment to the receptor[594,595].

Measurement of both the $G\alpha_s$–cAMP pathway and β-arrestin recruitment will be used as the two readouts for biased signalling. Whilst the GLP-1R does signal independently through other G proteins to induce Ca^{2+} flux and ERK1/2 phosphorylation, both the $G\alpha_s$ and β-arrestin pathways are associated with the primary effects elicited by activating both of these receptors.

3.1.5 Quantification of biased signalling

Bias can exist at the level of the receptor and system (cell or tissue) as well as the agonist[596] (See Introduction section 1.5). Consequently, biased agonism can only be

determined against a reference peptide. Bias can be intuitively observed. A ligand that has higher potency or affinity for one signalling pathway over another compared to a reference compound is instinctively biased toward the former pathway. However, complexities emerge when signalling efficacies and potencies diverge between ligands.

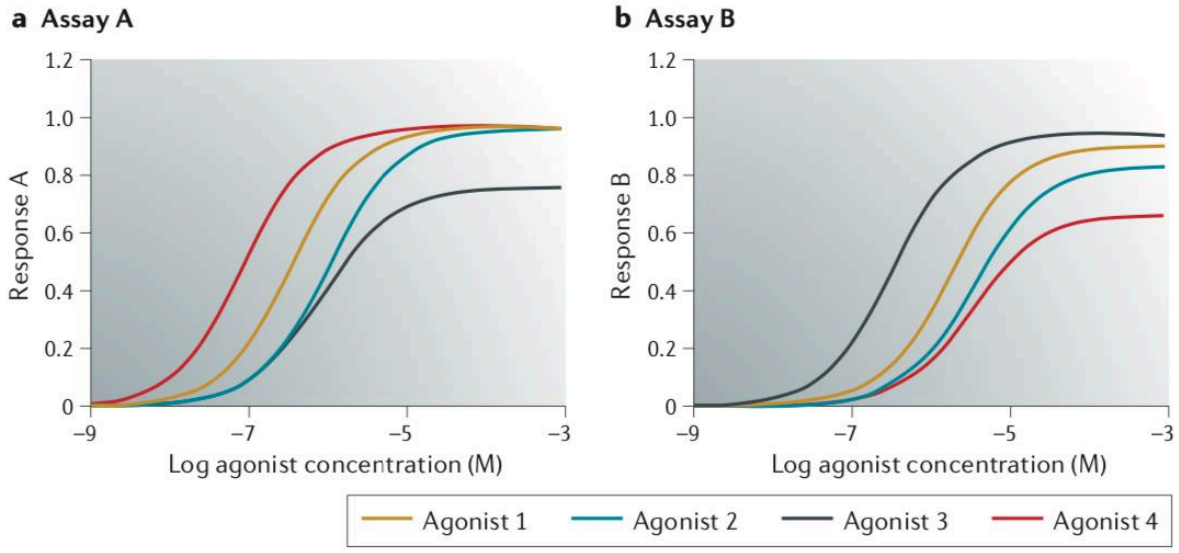

Figure 3.1 - Schematic of biased agonism between two signalling pathways

Bias can exist as a result of reduced efficacy or affinity. Agonist 4 (red) is visually biased toward assay A as it produces a greater maximum response compared to Agonist 1 (yellow). If Agonist 4 produced a maximum response but was still right shifted (as in Assay B) it would be biased toward Assay A based on affinity to the assay A. Figure adapted from Kenakin & Christopolous[3]

The most established method of bias quantification used in literature is the modified version of the operational model of pharmacological agonism[476]. Here, the transduction ratios (τ/K_a) are calculated for each intracellular pathway which combines receptor binding and coupling to that specific intracellular pathway with a transduction readout. Whilst the modified operational model accounts for changes in receptor and transducer density, one disadvantage of using the operational model is the inaccuracy of transductor ratio calculation for very partial agonists with little difference between baseline and maximum response[480]. Therefore, alternative methods have been proposed, including the relative activity scale ($\Delta\log E_{max}/EC_{50}$) which incorporates the estimated parameters E_{max} (efficacy) and EC_{50} (potency) to quantify bias at a receptor[477,478]. Note, however, that however this approach is only applicable when the Hill coefficient is one[597]. Finally, due to the inaccuracy of calculating τ/K_a values from

partial agonists, a further refinement of the operational model has been described which uses intrinsic antagonist actions of partial agonists to more accurately calculate transduction ratios[479], hereby called the “competitive method”. These three approaches are all used in the present work.

3.1.6 Aims

The aims in this chapter are:

1) Using an in-house peptide library, discover amino acid switches in dual GLP-1R/GCGR agonists which lead to alteration of cAMP or β-arrestin signalling at either receptor
2) Examine the acute and chronic effects of G protein- or β-arrestin-biased dual GLP-1R/GCGR agonists *in vitro*.

3.2 Results

3.2.1 Identification of biased dual GLP-1R/GCGR agonists

To identify biased dual GLP-1R/GCGR agonists, peptides were identified (parent compounds) with multiple corresponding compounds with single amino acid substitutions (Daughter compounds, Table 3.1), using the available peptide library. Candidate peptides were screened for unique receptor signalling properties using a single-dose β-arrestin recruitment assay in combination with historical cAMP data produced in the laboratory. Screening assays were performed at 1μM as this was sufficient to achieve recruitment with the endogenous ligands, therefore daughter compounds with reduced recruitment at 1μM could be assumed to be weak agonists for β-arrestin recruitment.

Parent	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Daughter 1	X	A	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Daughter 2	X	X	X	X	X	X	X	X	B	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Daughter 3	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	C	X	X	X	X	X

Table 3.1 - Schematic of "parent" peptides with corresponding "daughter" compounds with single amino acid substitutions

X – amino acid; A, B, C (red) – amino acid substitutions

The substitution of serine at position 2, native to both OXM and glucagon, to 2-aminoisobutyric acid (referred here as AIB2) was identified by this method and resulted in the reduction of β-arrestin recruitment of 74% at the GCGR compared to its parent (One-way ANOVA; $P<0.0001$) (Figure 3.2A, bottom) without significant effects on GLP-1R β-arrestin-2 recruitment ($P>0.05$) (top).

As cAMP produced in the $G_{\alpha s}$ – AC – cAMP signalling pathway does so in a cascadic manner, it is not possible to measure cAMP accumulation in a similar single-dose assay.

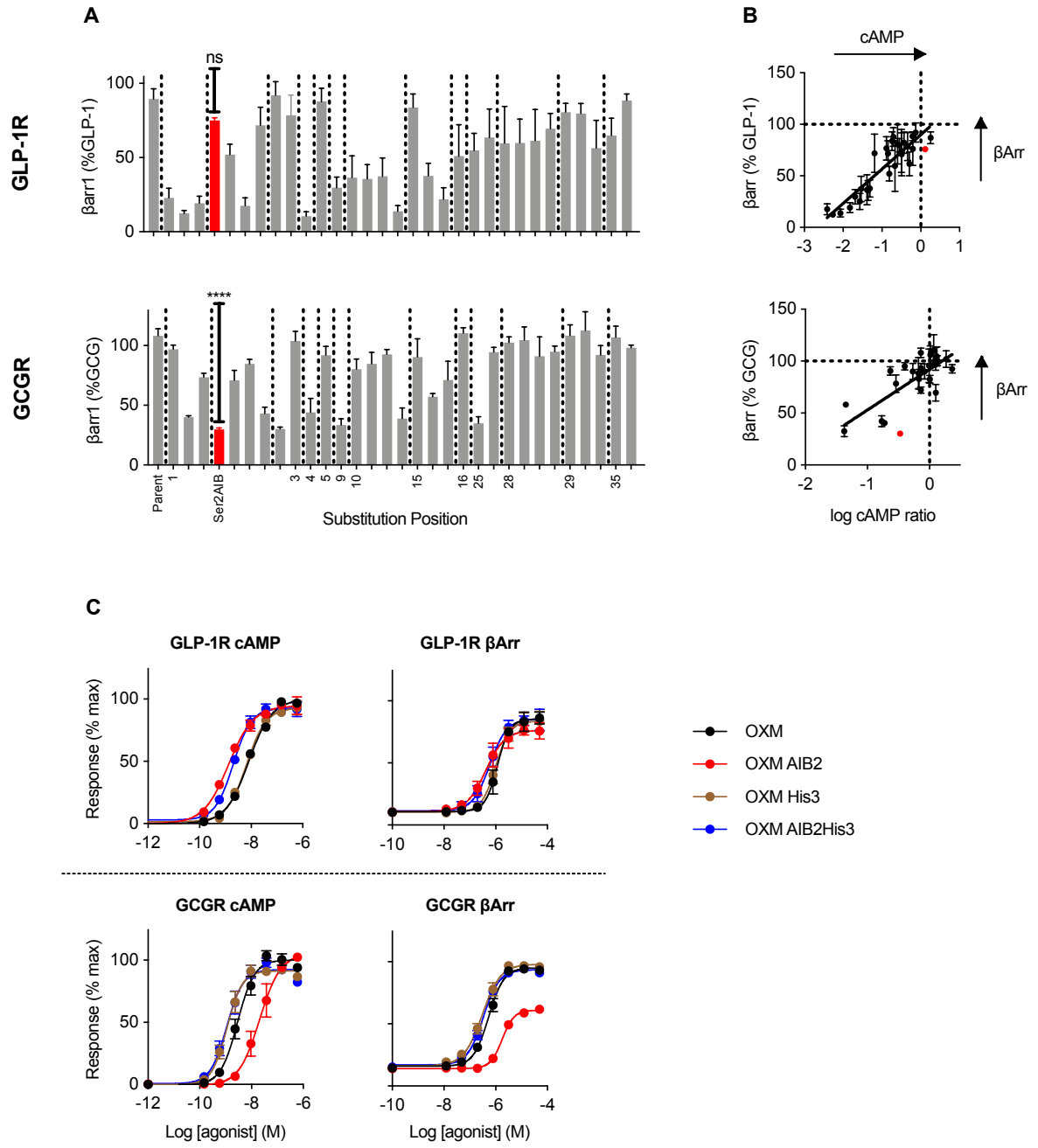

Figure 3.2 - Dose-response of oxyntomodulin derivatives for cAMP signalling and β-arrestin-2 recruitment

(A) Percentage of β-arrestin 1 recruitment in PathHunter® CHO-K1 cells expressing human GLP-1 receptor (top) or glucagon receptor (bottom) cells after a 30-minute stimulation of 1μM peptide (n=5). Results shown relative to native ligand (GLP-1 or glucagon). Data was analysed using One-Way ANOVA with Dunnett's test. (B) Regression analysis of β-arrestin 1 recruitment *versus* the normalised EC_{50} for cAMP generation at GLP-1 receptor (top) and glucagon receptor (bottom). All data for cAMP production produced in house and normalised relative to native ligand (GLP-1 or glucagon). (C) Dose response analysis of cAMP production (left) or β-arrestin 2 recruitment (right) by oxyntomodulin (OXM, black) or oxyntomodulin with 2-aminoisobutyric acid at position 2 (OXM-AIB2, red), histidine at position 3 (OXM-His3, gold) or AIB2 and His3 together (OXM-AIB2His3, blue) to PathHunter® CHO-K1 hGLP-1R (top row) or hGCGR (bottom row) after a 30-minute stimulation (n=5). Statistical significance performed by one-way ANOVA with Tukey post hoc test. Data presented as mean ± SEM.

For this reason, previously collected pEC_{50} values were used. The cAMP collected as part of the drug discovery process was collected as mean pEC50 values and the reference pEC50 subtracted from the pEC50 of the endogenous ligand (e.g. GLP-1 or GCG). Data from the single dose β-arrestin recruitment assay were then mapped against collected cAMP data as an XY regression plot to create a simple regression analysis (Figure 3.2B). Potent agonists would be expected to generate high β-arrestin recruitment and cAMP accumulation and *vice versa* for low potency/partial agonists. Here, addition of AIB2 maintained robust cAMP potency at the GCGR whilst having diminished β-arrestin recruitment, with no observable effects at the GLP-1R, highlighting an AIB2 compound as interesting to take forward to look at selective diminishment of the β-arrestin pathway at the GCGR.

Differential signalling effects at either pathway with addition of AIB2 were not replicated at the GLP-1R, suggesting the effect was specific to the GCGR. Interestingly, most therapeutic incretins currently on the market or in clinical trials contain the AIB2 switch, as this substitution protects the peptide from proteolytic degradation by dipeptidyl peptidase 4[598]. This means that not only would addition of AIB2 produce an agonist with reduced β-arrestin-2 recruitment specifically at the GCGR, such an agonist would be pharmacokinetically advantageous for use *in vivo*, increasing its therapeutic viability.

Previous work in the laboratory had suggested that the mild loss of cAMP signalling potency at the GCGR can be counteracted by substituting the native glutamine at position 3 of the compound (hereby called Gln3) with histidine (His3). To confirm the effect of AIB2 addition on β-arrestin recruitment and cAMP potency, and the effect of substituting in His3, native OXM was synthesised with systematic substitutions of AIB2, His3 and AIB2His3 (see Appendix). In CHO-K1 hGLP-1R-βArr2 cells, addition of AIB2, regardless of the third amino acid, resulted in an approximately five-fold increase in cAMP potency (Figure 3.2C, top left) with no discernible effect on β-arrestin recruitment (top right). At the GCGR, however, OXM-AIB2 (red) showed a seven-fold reduction in cAMP potency (bottom left) and a 40% reduction in β-arrestin recruitment compared to OXM (black, bottom right), both of which were completely rescued by addition of His3 (blue). His3 alone showed no significant difference from OXM in any

signalling pathways (brown), therefore signifying His3 by itself had no unique signalling properties. Thus, from this initial study, two peptides were identified containing AIB2Gln3 or AIB2His3 which could be used as a tool to further investigate the functional effects of β-arrestin signalling at the GCGR.

3.2.2 Bias quantification of pharmacokinetically-enhanced AIB2Gln3 and AIB2His3 dual agonists

Following the discovery of this "His/Gln" amino acid switch which controlled GCGR-mediated β-arrestin recruitment without invasive genetic manipulation, a pair of pharmacologically-superior AIB2Gln3/AIB2His3 compounds were synthesised. These "SRB" compounds contained N-terminal and C-terminal amino acid substitutions which enhanced the ligands potency at each receptor and their pharmacokinetic profile (see Appendix). In addition to this, four other AIB2His3/Gln3 pairs were identified to corroborate any consequent *in vitro* and *in vivo* results.

SRB103Gln3 and SRB103His3 were tested for their respective potency and efficacy for cAMP production and β-arrestin recruitment. Like the OXM peptides, SRB103Gln3 and SRB103His3 showed a near identical potency and efficacy for cAMP production and β-arrestin recruitment at the GLP-1R (Figure 3.3A, top row). At the GCGR, SRB103Gln3 and SRB103His3 showed identical potency for cAMP production (Figure 3.3A, bottom left), and in tandem with the previous results, SRB103Gln3 produced a significant 46% reduction of β-arrestin recruitment compared to SRB103His3 (two-way ANOVA, $P<0.001$) (Figure 3.3A, bottom right). These data were corroborated in the four further AIB2His3/Gln3 pairs, however the potency of the AIB2Gln3 compounds to cAMP production was worse than the AIB2His3 counterpart (Table 3.2). Interestingly, in all four other cases of AIB2His3/Gln3 peptide pairs, AIB2Gln3 peptides displayed significantly reduced β-arrestin-2 recruitment at the GLP-1R as well as at the GCGR compared to His3 comparators, due to His3 compounds acting as full agonists for β-arrestin recruitment.

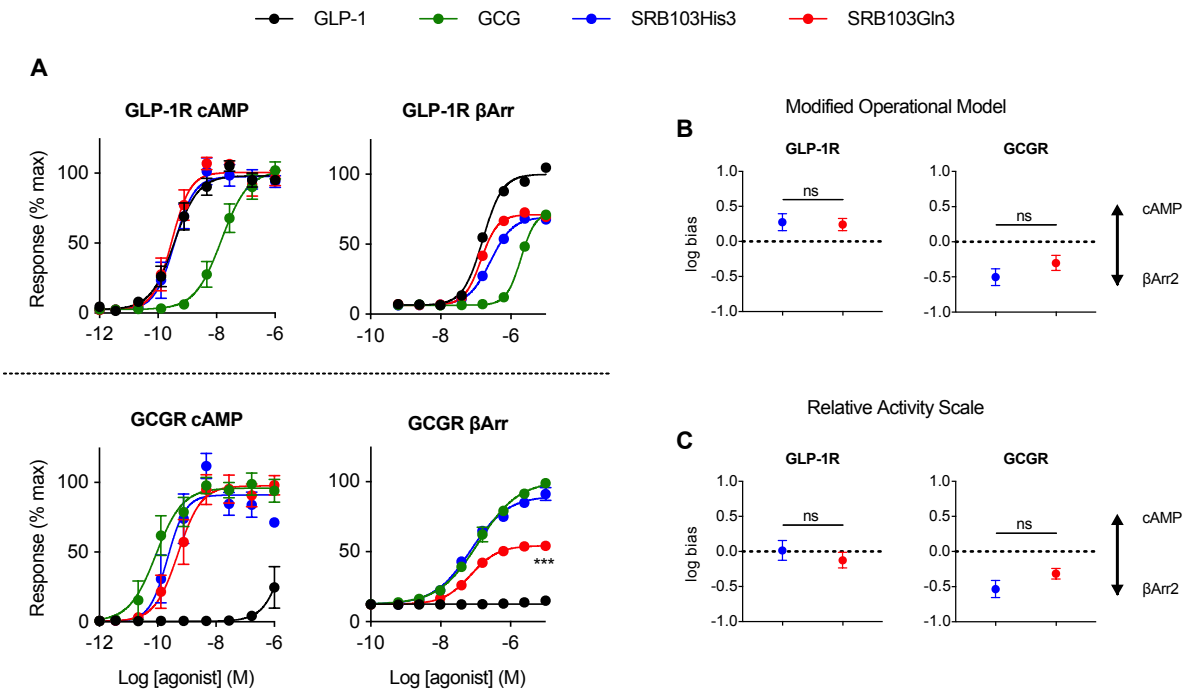

Figure 3.3 - Dose-response of SRB103 peptides for cAMP signalling and β-arrestin-2 recruitment

(A) Dose response of GLP-1 (black), glucagon (green), SRB103His3 (blue) and SRB103Gln3 (red) for cAMP signalling (left) or β arrestin-2 recruitment (right) in DiscoverX CHO-K1 cells expressing the GLP-1 receptor (top) or GCGR (bottom). Cells were stimulated for 30 minutes at 37°C (n=5-6). (B) Bias calculation ($\Delta\Delta\log(\tau/K_A)$) using modified operational model with SRB103His3 and SRB103Gln3 using GLP-1 or GCG as the reference compound. Calculation used: $\Delta\tau/K_{a[cAMP]} - \Delta\tau/K_{a[\beta Arr]}$. (C) As for (B) but using the relative activity scale, and the calculation used was $\Delta(EC_{50}/E_{max})_{[cAMP]} - \Delta(EC_{50}/E_{max})_{[\beta Arr]}$. Statistical significance of E_{max} (A) calculated using two-way ANOVA with Tukey post hoc test, and bias using student t-test. Error bars show SEM. *** P<0.001; ns – not significant.

Having observed apparent signalling bias shown by SRB103Gln3 toward cAMP signalling at the GCGR, accepted methods of pharmacological bias were used to confirm and quantify this. Here, bias to each signalling pathway was measured relative to GLP-1 or GCG. Surprisingly, the modified operational model, commonly used to distinguish biased agonists, did not certify SRB103Gln3 as a biased agonist at the GCGR relative to GCG or SRB103His3 (student t-test; P>0.05) (Figure 3.3B, right), despite the clear visual reduction in β-arrestin-2 recruitment elicited by SRB103Gln3 stimulation. Interestingly, both SRB103 compounds shows slight preference for β-arrestin-2 recruitment over cAMP at the GCGR, compared to GCG. SRB103His3 was more "β-arrestin-2-preferring" suggesting SRB103Gln3 may have a slight bias toward cAMP compared to SRB103His3. No difference was observed at the GLP-1R either (P>0.05) (Figure 3.3B, left). Conversely, both SRB103 peptides showed a very mild preference for cAMP signalling at the GLP-1R compared to GLP-1. Similarly, the relative activity scale showed no significant difference in bias between peptides

(P>0.05), but SRB103Gln3 showed a preference for cAMP signalling compared to SRB103His3, as expected (Figure 3.3C). Results were replicated in four additional versions of His3/Gln3 peptides (Table 3.3). Here, a separate attempt to improve the accuracy of the transduction ratio (τ/K_A) was made by using the "competitive analysis" model, however this method also failed to confirm Gln3 peptides as "cAMP-biased".

GLP-1R								
Peptide	His1	Gln1	His2	Gln2	His3	Gln3	His4	Gln4
cAMP pEC50	9.37 ± 0.2	9.22 ± 0.2	9.28 ± 0.2	9.26 ± 0.2	9.23 ± 0.2	9.14 ± 0.2	9.18 ± 0.1	9.06 ± 0.2
BArr pEC50	6.26 ± 0.1	6.12 ± 0.1	6.34 ± 0.1	6.09 ± 0.2	6.28 ± 0.1	6.25 ± 0.1	6.35 ± 0.1	6.20 ± 0.1
BArr Emax	109.0 ± 2.8	58.5 ± 2.1	109.5 ± 6.8	69.5 ± 3.7	111.1 ± 4.5	66.3 ± 3.3	98.3 ± 6.0	55.4 ± 4.4
GCGR								
Peptide	His1	Gln1	His2	Gln2	His3	Gln3	His4	Gln4
cAMP pEC50	10.00 ± 0.2	9.30 ± 0.2	9.84 ± 0.2	9.37 ± 0.2	9.81 ± 0.3	9.52 ± 0.2	9.82 ± 0.2	9.47 ± 0.2
BArr pEC50	7.13 ± 0.1	6.63 ± 0.2	7.12 ± 0.0	6.7 ± 0.2	7.00 ± 0.1	7.05 ± 0.0	7.04 ± 0.0	6.89 ± 0.1
BArr Emax	109.7 ± 5.4	41.7 ± 0.9	98.6 ± 1.8	38.8 ± 6.9	106.1 ± 2.6	41.7 ± 5.1	97.6 ± 4.8	36.9 ± 4.3

Table 3.2 - Affinity and efficacy of four other His3/Gln3 peptide pairs

Data presented as mean ± SEM. Data normalised to GLP-1/glucagon E_{max}.

Operational model								
Peptide	His1	Gln1	His2	Gln2	His3	Gln3	His4	Gln4
GLP bias	0.109 ± 0.27	0.359 ± 0.27	-0.105 ± 0.21	0.035 ± 0.28	0.121 ± 0.19	0.424 ± 0.25	-0.260 ± 0.23	0.250 ± 0.25
GCG bias	0.129 ± 0.10	0.313 ± 0.25	0.006 ± 0.14	-0.038 ± 0.18	0.090 ± 0.06	0.230 ± 0.22	-0.007 ± 0.06	0.299 + 0.21
Competitive analysis								
Peptide	His1	Gln1	His2	Gln2	His3	Gln3	His4	Gln4
GCG bias	0.129 ± 0.10	0.348 ± 0.17	0.006 ± 0.14	0.085 ± 0.14	0.090 ± 0.06	0.192 ± 0.13	-0.007 ± 0.06	0.116 ± 0.09
Relative activity scale								
Peptide	His1	Gln1	His2	Gln2	His3	Gln3	His4	Gln4
GLP bias	0.129 ± 0.12	0.403 ± 0.20	0.009 ± 0.20	-0.185 ± 0.32	-0.035 ± 0.16	0.164 ± 0.13	0.318 ± 0.28	0.021 ± 0.19
GCG bias	0.134 ± 0.07	0.413 ± 0.24	-0.113 ± 0.13	0.274 ± 0.18	0.022 ± 0.15	0.140 ± 0.116	-0.045 ± 0.07	0.168 ± 0.16

Table 3.3 - Bias quantification between four other AIB2Gln3/His3 peptide pairs

Quantification performed using three methods; modified operational model, "competitive analysis" and relative reactivity scale. No statistical difference observed for any pair (P>0.05), using one-way ANOVA. Data shows mean ± SEM.

3.2.3 Measurement of G protein and β-arrestin recruitment

Whilst initial data demonstrated all AIB2Gln3 peptides tested as showing signal bias toward cAMP-accumulation compared to β-arrestin recruitment, this was not

confirmed by mathematical quantification typically used to confirm biased signalling. This could be because cAMP production is a cascadic pathway, where a single phosphorylated AC can catalyse the production of many-fold more cAMP molecules. For this reason, sub-maximal $G\alpha_s$ recruitment, which results in sub-maximal AC phosphorylation, might still produce the maximum amount of cAMP possible in the system. Recruitment kinetics of $G\alpha_s$ was therefore investigated to explore this paradigm.

HEK293T cells were co-transfected with either the hGLP-1R-SmBit or hGCGR-SmBit plasmid alongside either a MiniG-LgBit or β-arrestin-2-LgBit plasmid to quantify recruitment dynamics of MiniGα_s or β-arrestin-2 to the receptor at increasing concentrations of SRB103 peptides. Of note, at the GLP-1R both SRB103 peptides produced and approximate eighteen to twenty-fold increase in MiniGα_s recruitment at the peak response (Figure 3.4A, top row). However, SRB103Gln3 produced approximately half the peak response of β-arrestin-2 recruitment compared to SRB103His3 (bottom row). This suggests that, contrary to data in figure 3.3, SRB103Gln3 is unable to fully initiate β-arrestin-2 recruitment to the GLP-1R. Profiles at the GCGR, unusually, between the two peptides were quite similar, with SRB103Gln3 (Figure 3.4B, top row) showing a very mild reduction in maximal recruitment at both pathways compared to SRB103His3 (bottom row).

Area under curve (AUC) was calculated for each curve produced by the corresponding concentration of peptide (Figure 3.4C). Using the AUC of recruitment at 10μM peptide stimulation as a surrogate for maximal MiniG$_s$ or β-arrestin-2 recruitment, SRB103Gln3 produced a sub-maximal amount of β-arrestin-2 recruitment at the GLP-1R ($P<0.01$), but no difference in MiniGs recruitment ($P>0.05$), compared to SRB103His3. At the GCGR, SRB103Gln3 reduced both β-arrestin-2 $P<0.05$) and MiniG$_s$ ($P<0.01$) recruitment compared to SRB103His3 (Figure 3.4D). These data suggest that, rather than selectively signalling for cAMP at the GCGR by displaying

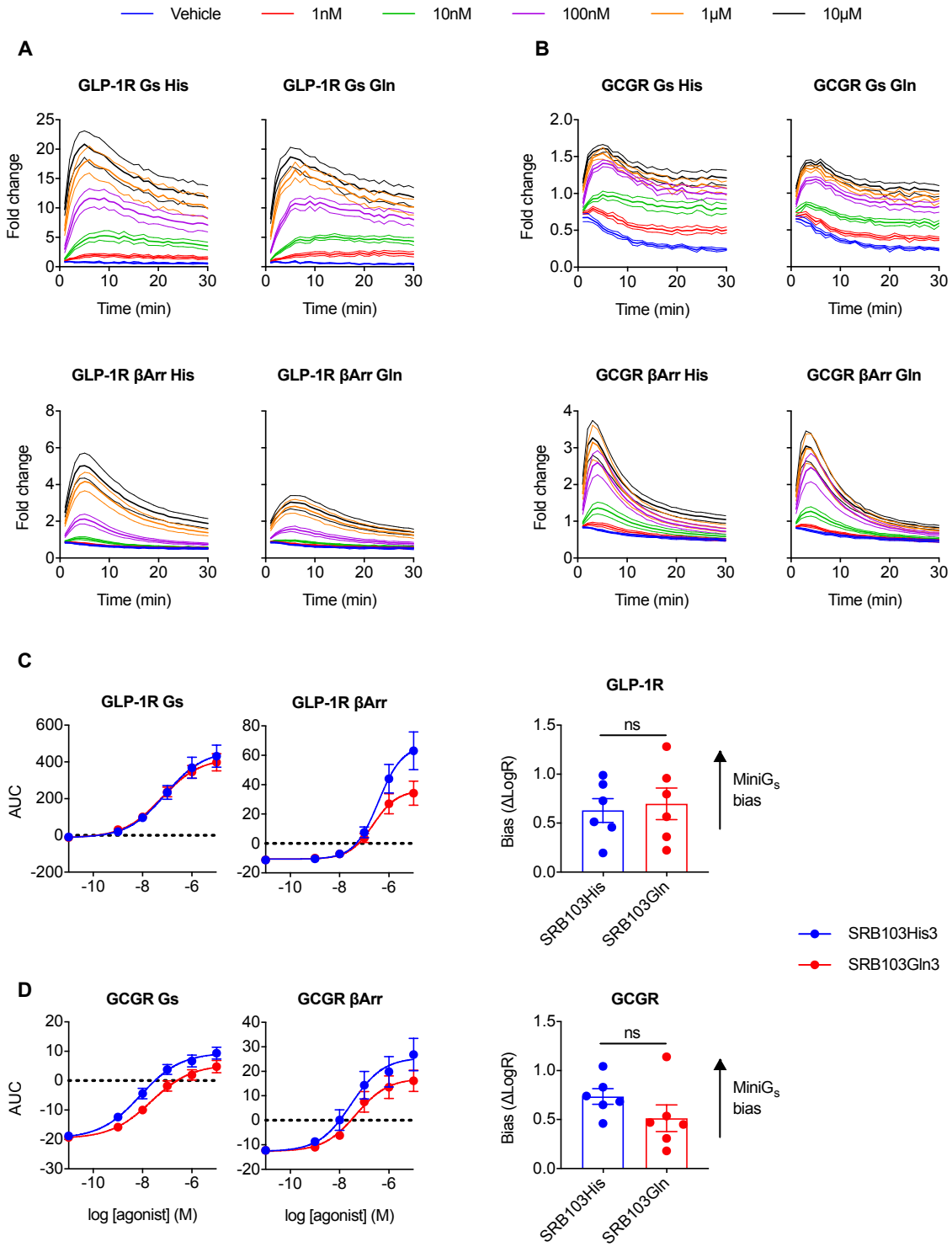

Figure 3.4 - NanoBit measurement of the recruitment of MiniGs or β-arrestin-2 to the GLP-1R or GCGR

(A) Recruitment dynamics of MiniGs-LgBit (top row) or βArr-LgBit.(bottom row) to GLP-1R-SmBit across a 30-minute stimulation with SRB103His3 (left) or SRB103Gln3 (right) in transiently transfected HEK293T cells. Concentrations used were 1nM (red), 10nM (green), 100nM (magenta), 1μM (orange), 10μM (black) or vehicle (blue). Data shown as fold change from baseline, which was the mean average recruitment for 5 minutes before peptide stimulation. (B) as for (A) but in the hGCGR. (C) Area under curve (AUC) of MiniGs (left) or β-arrestin-2 (middle) recruitment curves generated in (A), after 30-minute stimulation by SRB103His3 (blue) or SRB103Gln3 (red). ΔLogR ($LogR_{[Gαs]} – LogR_{[βArr2]}$) values for each replicate then calculated from curves to determine bias toward either pathway (right) (D) As for (C) but in the hGCGR. N=6. Statistical analysis of bias between SRB103 peptides performed by student t-test. Data shown as mean ± SEM.

low efficacy for β-arrestin recruitment as suggested in section 3.2.2, this phenotype may in fact be present at the GLP-1R, whilst acting as a partial agonist at the GCGR. The LogR values of each AUC curve fitting were then analysed, and bias calculated by subtracting the LogR[β-arrestin] from LogR[$G\alpha_s$] for each assay (Figure 3.4C & D, right). Here, the higher the number, the greater the tendency for the agonist to recruit $MiniG_s$ over β-arrestin-2. Whilst no results were significant between peptides at each receptor (student t-test; $P>0.05$), SRB103Gln3 did show a reduced preference for MiniGs recruitment at the GCGR compared to SRB103His3 (Figure 3.4D, right), suggesting SRB103Gln3 shows bias toward β-arrestin-2 recruitment than MiniGs recruitment at the GCGR, contradicting results from before. No difference in bias was observed between the two peptides at the GLP-1R (Figure 3.4C, right).

3.2.4 cAMP accumulation upon prolonged receptor stimulation

It was recently reported that a GLP-1R agonists showing reduced cAMP potency but diminished β-arrestin recruitment resulted in a greater cAMP potency and efficacy than one with full β-arrestin recruitment after prolonged stimulation period[276]. This follows convention, that β-arrestin recruitment ultimately results in receptor internalisation. It is theorised that, if a receptor is stimulated for a sustained period of time by an agonist that does not induce full β-arrestin recruitment, more receptor will be available for stimulation at timepoints where, normally, a large proportion of the receptor has been internalised, resulting in greater cAMP accumulation.

To confirm that this paradigm translates into SRB103Gln3, which showed diminished β-arrestin recruitment at the GCGR in both the PathHunter and NanoBit systems, and decipher which result was correct for the GLP-1R, cells overexpressing either the GCGR or GLP-1R were stimulated for 30 minutes or 16 hours to discover whether a divergence in cAMP signalling could be uncovered upon prolonged receptor activation. If reduced β-arrestin recruitment did prolong GCGR signalling, this would emulate what is seen at the GLP-1R[276].

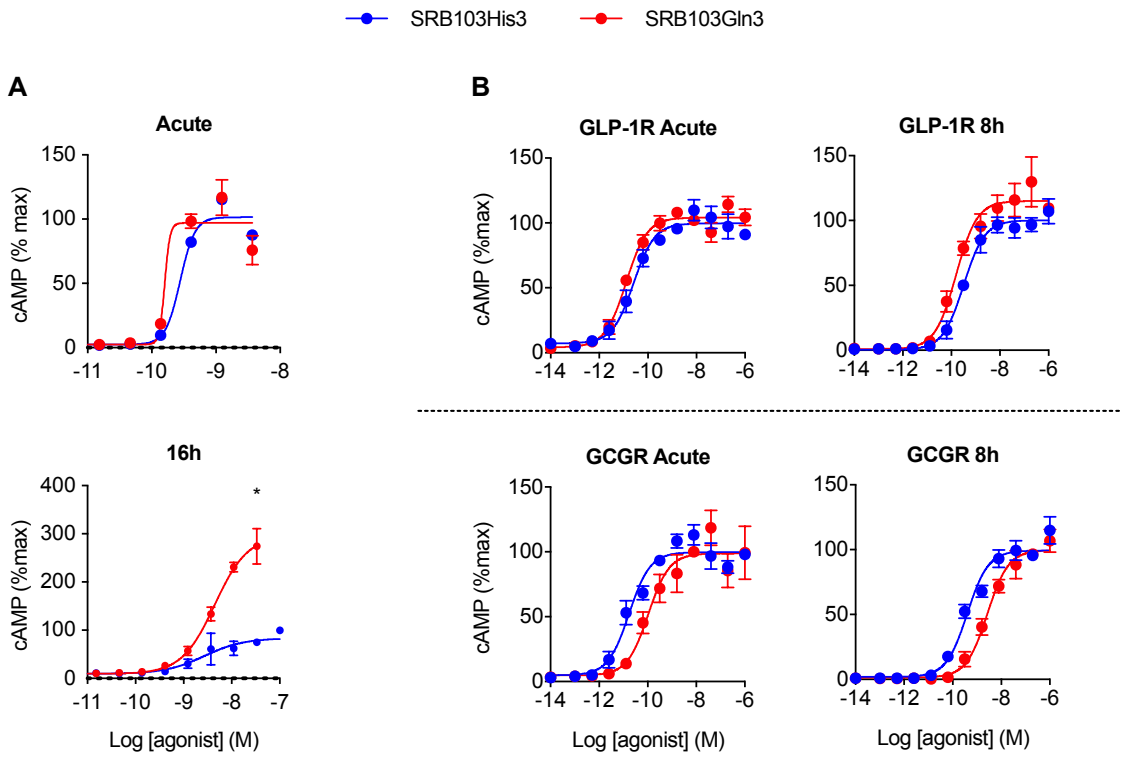

Figure 3.5 - Effect of prolonged stimulation of the GCGR or GLP-1R with SRB103 peptides

(A) Human hepatoma (Huh7) cells expressing hGCGR were stimulated with SRB103Gln3 (red) or SRB103His3 (blue) in the presence of 500μM IBMX for 30 minutes (left) or 16 hours (right) to measure cAMP production. * P<0.05 by paired t test comparing E_{max}. Data expressed relative to SRB103His3 Emax, n=3. (B) HEK293T cells were transfected overnight with GLP-1R (top row) or GCGR (bottom row) and stimulated for either 30 minutes (left) or 8 hours (right) in the presence of 500μM IBMX. Data expressed relative to SRB103His3 E_{max}. N=3. Statistical analysis of E_{max} performed by two-way ANOVA with Tukey post hoc test. Data shown as mean ± SEM. *P<0.05.

Firstly, Huh7 hepatoma cells over-expressing the human GCGR were stimulated with SRB103 peptides. Here, both peptides showed equal potency to the receptor after 30 minutes of stimulation (Figure 3.5A, top). However, clear divergences appeared after 16 hours of stimulation, where G103Gln produced 3.6 times more cAMP than SRB103His3 at peak cAMP response (two-way ANOVA; P>0.05) (Figure 3.5A, bottom).

To confirm whether this effect from SRB103Gln3 was unique to the GCGR, or whether the GLP-1R also showed prolongation of signalling upon SRB103Gln3 stimulation, HEK293T cells were transfected with either the SNAP-tagged GLP-1R or GCGR overnight and then treated for either 30 minutes or 8 hours with either SRB103Gln3 or SRB103His3. Interestingly, this showed no divergence between stimulation time

points (Figure 4B). To confirm that this was not due to the high expression of the receptor masking any nuanced changes in signalling, transfected receptor mRNA was diluted in pcDNA. This had no effect on cAMP potency or efficacy (Table 3.4). These data suggest that there are system-specific variations in prolonged signalling effects elicited by SRB103Gln3, which require further investigation.

	GLP-1R Acute					
	1:5		1:25		1:125	
	Emax	pEC50	Emax	pEC50	Emax	pEC50
SRB103His	100	10.1 ± 0.5	100	10.9 ± 0.2	100	10.1 ± 0.1
SRB103Gln	85.8 ± 21.8	9.3 ± 1.0	98.9 ± 7.8	10.3 ± 0.2	78.2 ± 12.6	10.2 ± 0.4
	GLP-1R Chronic					
	1:5		1:25		1:125	
	Emax	pEC50	Emax	pEC50	Emax	pEC50
SRB103His	100	9.4 ± 0.0	100	9.2 ± 0.1	100	8.8 ± 0.2
SRB103Gln	105.5 ± 7.5	9.5 ± 0.3	101.6 ± 1.5	9.4 ± 0.3	101.8 ± 0.8	9.2 ± 0.1
	GCGR Acute					
	1:5		1:25		1:125	
	Emax	pEC50	Emax	pEC50	Emax	pEC50
SRB103His	100	10.4 ± 0.2	100	10.2 ± 0.1	100	9.7 ± 0.3
SRB103Gln	126.8 ± 16.4	9.5 ± 0.0	108.4 ± 6.4	9.4 ± 0.1	83.6 ± 7.6	8.8 ± 0.0
	GCGR Chronic					
	1:5		1:25		1:125	
	Emax	pEC50	Emax	pEC50	Emax	pEC50
SRB103His	100	9.0 ± 0.2	100	8.7 ± 0.2	100	8.6 ± 0.1
SRB103Gln	86.5 ± 3.6	8.4 ± 0.2	93.9 ± 7.9	8.0 ± 0.1	102.5 ± 19.4	7.8 ± 0.1

Table 3.4 - cAMP potency and efficacy in HEK293T cells transfected with GLP-1R or GCGR diluted in pcDNA

Cells transfected for 16 hours then stimulated for 30 minutes. Data normalised to SRB103His3 E_{max}.

3.2.5 Visualisation of GLP-1R and GCGR internalisation upon SRB103 compound stimulation

Results from the chronic stimulation of GCGR-containing Huh7 cells clearly indicate that SRB103Gln3 is able to maintain cAMP signalling after an overnight stimulation, unlike SRB103His3. One of the essential effects mediated by β-arrestin recruitment which could affect receptor signalling over 16 hours of stimulation is receptor internalisation, which was therefore investigated in further detail.

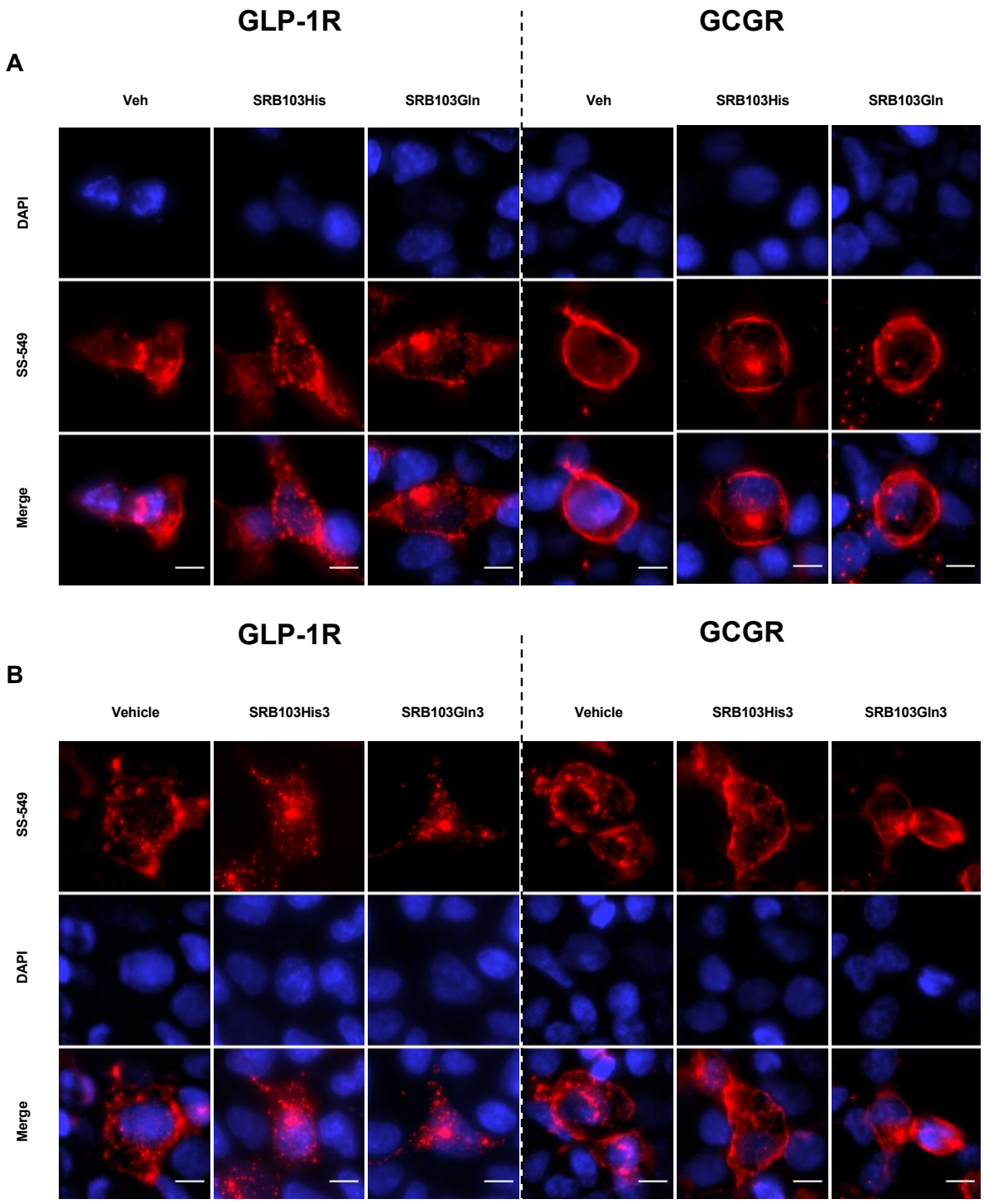

Figure 3.6 - GLP-1R and GCGR internalisation after prolonged stimulation by SRB103 peptides

(A) Representative images showing internalisation of SNAP-GLP-1R (left) or SNAP-GCGR (right) labelled with SNAP-Surface 549 (SS-549, red) after a 30-minute stimulation with 100nM SRB103 peptide or vehicle. Nuclear marker DAPI shown in blue. (B) as for (A) but with 4-hour stimulation. N=3/4, scale bar represents 8μm.

Here, HEK293T cells were transfected with either the human SNAP-tagged GLP-1R or GCGR and treated with a fluorescent probe which conjugates to the SNAP tag on the receptor. Selecting representative images of fixed cells, it was not possible to visually distinguish between receptor internalisation caused by SRB103Gln3 or SRB103His3 after acute 30 minute agonist stimulation (Figure 3.6A) or after a chronic 4 hour stimulation (Figure 3.6B).

Disparities in receptor internalisation were not observed, suggesting SRB103Gln3 may not be eliciting it's effects by reducing receptor internalisation rates. It is also important, in the future, to consider the wider aspect of β-arrestin effects when examining the mechanisms behind biased signalling beyond receptor internalisation toward desensitisation and non-canonical signalling pathways which may be activated by β-arrestin complexes, such as ERK1/2 and CREB pathways[275,280].

3.3 Discussion

In this chapter, novel biased dual GLP-1R/GCGR agonists were identified and characterised *in vitro* using a variety of assays. The key findings of this chapter are:

1) Novel switch of the third amino acid from His to Gln results in diminished β-arrestin recruitment, with little effect on cAMP potency.
2) SRB103Gln3 displays low efficacy for β-arrestin recruitment specifically at the GCGR and a standard full agonist at the GLP-1R in the CHO cell assays, but a partial agonist at the GCGR and low efficacy β-arrestin ligand agonist at the GLP-1R in the NanoBiT assays.
3) The three bias quantification methods used fail to identify SRB103Gln3 as biased at either receptor, despite clear differences in signalling profiles between the SRB103 peptides in both the DiscoverX and NanoBit assays.
4) Prolonged signalling of SRB103Gln3 in hepatoma cells overexpressing GCGR results in amplified cAMP production. This is not replicated in a GLP-1R model.
5) No visual differences in GCGR or GLP-1R internalisation are observed between either peptide after either 30 minutes or 4 hours of receptor stimulation.

3.3.1 Biased dual agonists identified using medium-throughput screen

In this chapter, dual agonists with differing signalling profiles for cAMP accumulation and β-arrestin recruitment were identified using a medium throughput screen (Figure 3.2). The medium throughput screen allowed identification of peptides based on β-arrestin recruitment, a surrogate for intrinsic receptor efficacy.

Interestingly, only one amino acid switch was taken forward for investigation, due to a number of possibilities. Firstly, the peptide library available contained compounds with very similar pharmacological profiles, with differences primarily designed to affect the pharmacokinetics and physicochemical nature of the peptide as opposed to producing interesting pharmacology. All peptides are from a drug development library where therapeutic performance and drug-like properties are of paramount importance. GLP-1R mono-agonists leading the field for anti-diabetic and anti-obesity therapeutics, require a once weekly injection[599]. Semaglutide was recently approved by the FDA for

use as a once-daily oral pill[600], and is expected to be a great success as it improves patient compliance. This highlights how a major driver in the field of incretin therapies is improving pharmacokinetics and delivery of the drug, rather than only pharmacodynamics.

Whilst time- and resource-efficient, using a single high dose to identify compounds with differing efficacies came at the likely expense of omitting compounds with low *potency* for β-arrestin recruitment. It is entirely possible that "daughter peptides" were missed which displayed lower potency for β-arrestin recruitment, but still produced full recruitment at the 1μM concentration used. The historical data used to derive cAMP potency was not performed in tandem with the parent compound, which increases the intra-assay variability of the data.

Nevertheless, the Gln3 to His3 amino acid switch found in this screening assay was robust throughout multiple examples of peptides with single Gln3 to His3 substitutions (Table 3.2). This suggests the assay is a fast and reliable method of detecting future compounds which may exhibit biased signalling properties at either receptor. A more rigorous method of analysing bias across the dual agonists could have been to perform alanine screen mutagenesis, where amino acids are systematically replaced by alanine along the molecule. The R-group of alanine is a small, non-polar methyl group and therefore ablates the specific interactions made by the native amino acid, whilst being less likely to affect overall peptide structural integrity. Alanine screening is regularly used to interrogate the stability, signalling and ligand interactions in GPCRs, including both the GLP-1R and GCGR[601-605]. Such a technique could, therefore, be used to interrogate amino acid interactions responsible for eliciting selective signalling for the SRB103 peptides at either receptor.

3.3.2 System differences and ligand-receptor interactions responsible for biased signalling

One of the central aims of this chapter was to identify and validate biased dual GLP-1R/GCGR agonists. Substitution of AIB in the N-terminus of Ex4 results in reduced β-arrestin signalling at the GLP-1R[276]. Screening dual agonists revealed this substitution had a marked affect at the GCGR, however effects are less discernible at the GLP-1R

compared to the Ex4 analogues (Figure 3.3A, bottom right). This suggests conformational differences between the SRB103 compounds *versus* the Ex4 analogues when complexed to the GLP-1R. This mimics the recent paradigm that separate ligands binding to a receptor can induce distinct receptor conformations, which results in unique intracellular signalling patterns[254,606-608]. Similarly, in the NanoBit assay, the substitutions in SRB103Gln3 resulted in a full agonist for $G\alpha_s$ recruitment but partial β-arrestin recruitment at the GLP-1R, and partial agonist at the GCGR (Figure 3.4C & D), suggesting that amino acid interactions between the ligand and receptor drive the conformational change of the receptor. Switching glutamine, a large, neutral amino acid to histidine, a positively charged amino acid (at certain pHs) with a large aromatic ring will undoubtedly affect ligand-receptor interactions. Considering glutamine at position 3 of glucagon has been described as critical for receptor binding and activation, it is not surprising that this amino acid switch has considerable implications for GCGR signalling[609].

Whilst confirming the paradigm that N-terminal substitutions affect both GLP-1R and GCGR signalling, to what extent remains unresolved. Using the G_s Dynamic Kit to measure cAMP accumulation in CHO-K1 cells, SRB103Gln3 and SRB103His3 are nearly identical in potency for cAMP production at both receptors (Figure 3.3A, bottom left). However, measurement of direct MiniG protein recruitment in HEK293 cells indicated that SRB103Gln3 acted as a partial agonist for $G\alpha_s$ recruitment at the GCGR relative to SRB103His3 (Figure 3.4D, left), but full agonist for $G\alpha_s$ recruitment at the GLP-1R. This observation is explained by the inherent amplification in the $G\alpha_s$/cAMP/PKA signalling pathway, but raises questions about which readout is most appropriate. It may be that $G\alpha_s$ responses are more appropriate for understanding how different ligands drive conformational change that lead to altered interactions with intracellular effectors, but cAMP is more relevant for gaining a fuller picture of functionally important signalling responses within the cell.

3.3.3 Quantification analysis fails to qualify bias of SRB103Gln3 or SRB103His3

Mathematical quantification is used in the process of discovery of biased agonists, allowing for validation and comparison of the signalling of ligands to a receptor

irrespective of factors which affect assay to assay variation[3,476]. In this work, three separate analyses were used to calculate the bias shown by the SRB103 compounds, and four other versions with similar amino acid substitutions.

Firstly, the modified operational model was used to interrogate whether SRB103Gln3 showed signalling bias at either receptor compared to SRB103His3 (Figure 3.3B). SRB103His3 showed similar β-arrestin and cAMP signalling efficacies as the endogenous ligand at both the GLP-1R and GCGR, differing only in β-arrestin recruitment at the GCGR. Therefore, SRB103His3 was considered a suitable reference peptide. Despite clear differences in β-arrestin-2 recruitment seen specifically at the GCGR, the modified operational model could not distinguish SRB103Gln3 as being cAMP-biased at the GCGR compared to SRB103His3. There are two possible options why bias could not be calculated with this method. Firstly, the modified operational model has known limitations when quantifying very partial agonists, due to the inherent imprecision of calculating the transduction ratio when the baseline and maximum response are similar[479,596]. As SRB103Gln3, and the four corresponding AIB2Gln3 compounds show partial activity for β-arrestin recruitment at the GCGR (Table 3.2), this may result in inaccurate calculation of the transduction ratios for these compounds. Secondly, the pEC_{50} values for the SRB103 compounds, and other His3/Gln3 pairs tested are strikingly similar (7.15 and 7.14 for SRB103Gln3 and His respectively). Potency to the pathway is the key attribute driving transduction ratio calculation, and therefore with near identical potencies to all signalling pathways tested, it may be unsurprising this method failed to quantify bias.

Further alternative approaches were used to improve the definition of the transduction values for SRB103Gln3 at the GCGR, such as the “competitive method” (Table 3.3) and use different pharmacological parameters to further clarify potential bias with the relative activity scale (Figure 3.3C and Table 3.3). Both the relative activity scale and competitive analyses were not successful in confirming biased signalling. As the NanoBiT data suggest, SRB103Gln3 may in fact be a weak partial agonist at the GCGR with reduced efficacy and potency to both pathways tested. This contrasts with the cAMP assay data which suggests SRB103Gln3 can fully activate cAMP accumulation with similar potency and efficacy to SRB103His3Such differences could partly reflect the amplification inherent to the cAMP signalling pathway, but may also

be partly responsible from differences in host cell type (i.e. CHO-K1 *versus* HEK293T), receptor expression levels relating to stable *versus* transient transfection of receptor constructs, receptor N- and C-terminal tags and other factors.

Other methods of bias quantification are available. An alternative method used to quantify bias elicited by very partial agonists uses irreversible antagonists to highlight agonist efficacy to the receptor[610]. As there is no irreversible antagonist for the GCGR commercially available, this assay is currently unavailable to study biased partial agonists at the GCGR. This could be circumvented using a tetracycline-inducible knockout system to systematically titrate receptor expression[611,612].

Despite no mathematical evidence of SRB103Gln3 showing biased signalling, there was still compelling evidence that SRB103Gln3 had unique signalling properties at both receptors which was corrected by substitution of Gln3 to His3, meaning the SRB103 peptides were compelling to take forward into further testing.

3.3.4 SRB103Gln3 causes prolonged signalling at GCGR, not due to decreased internalisation

As β-arrestin is associated with receptor internalisation upon prolonged ligand stimulation, peptides with differential β-arrestin recruitment profiles can diverge in their ability to induce receptor internalisation[613]. It was recently shown that a weak agonist at the GLP-1R was paradoxically able to induce greater cAMP production with longer stimulation timepoints, due partly to reduced rates of receptor internalisation[276]. In the present study, dual agonists with the AIB2Gln3 substitution repeatedly showed reduced β-arrestin recruitment at both receptors with mitigated effects on acute cAMP production. This is the first discussed example of a peptide agonist displaying selective signalling for cAMP production with reduced β-arrestin recruitment at the GCGR, and the first example of dual GLP-1R/GCGR agonists screened for altered receptor signalling profiles.

In Huh7 hepatoma cell lines expressing the GCGR, all AIB2Gln3 peptides tested showed similar potency and efficacy for acute cAMP production compared to their AIB2His3 comparator (Figure 3.5A, top). However, noticeable divergences in potency

and efficacy between compounds occurred after 16 hours of peptide stimulation (Figure 3.5A, bottom). The hypothesis for this difference is that the reduction in recruitment of β-arrestins to the receptor caused by AIB2Gln3 peptides will reduce desensitisation and internalisation of the receptor. This in turn allows for prolongation of signalling, and hence improvement of cAMP production over greater timepoints. This fits with previous data showing that depletion of β-arrestin 2 increases hepatic GCGR signalling in primary hepatocytes[283], and suggests that peptides signalling at the GCGR can modulate the kinetics and strength of signal through β-arrestin recruitment kinetics. This is also exciting as the chronic stimulation of receptors mimics the therapeutic setting, whereby peptide with optimised pharmacokinetics will remain present in the plasma for at least a day. If it is possible to increase efficacy by reducing β-arrestin recruitment, as is suggested in these results, this may be an alternative to increasing potency of peptides to improve their therapeutic use.

It is clear, however, that these findings do not translate to the transfected HEK293T cells used to interrogate chronic stimulation of both receptors (Figure 3.5B). It could be hypothesised that differences seen in transfected cells may be due to a greater overexpression of receptor compared to a stable cell line, however receptor mRNA dilution had no effect on cAMP potency or efficacy at 8-hour stimulation periods (Table 3.4). One possible explanation for the lack of translation in the two cell lines could relate to the idea of system bias[3,596]. This results from iterations in intracellular machinery which could increase the effect of β-arrestin-mediated events. In Huh7 cells, this could include increased efficacy for recruiting GRKs, which phosphorylate the intracellular domain of the receptor to induce β-arrestin recruitment[614]. Of note, expression of GRKs in HEK293T cells is relatively low, with GRK2 reportedly undetectable[615]. As the GCGR is known to rapidly recycle back to the cell membrane after internalisation[403], perhaps there is a dampening of the recycling process in Huh7 cells which exaggerates any effects on internalisation. Alternatively, as the HEK293T cells were transfected with SNAP tagged receptors to replicate internalisation data shown in section 3.2.5, the ability of the GCGR or GLP-1R to activate β-arrestins in a physiological manner might be compromised. This is unlikely as evidence suggests the SNAP tag does not compromise cAMP potency[616], including for the GLP-1R[617], although this has not been confirmed for the GCGR.

One explanation for the results seen in the Huh7 cells is that AIB2Gln3 peptides could reduce the amount of GCGR internalisation. By labelling both the GLP-1R and GCGR with a fluorescent probe, receptor internalisation was visualised after SRB103 peptide stimulation under a fluorescent microscope. No differences in either GCGR or GLP-1R internalisation could be measured, suggesting that the prolonged signalling effect caused by reduced β-arrestin recruitment was not linked to effects on receptor internalisation. This was not a quantitative assay, meaning only gross differences were likely to be detectable. Internalisation of GCGR is also much more transient in comparison to other GPCRs[403] and has been seen to only occur at high ligand concentrations[618]. A number of further approaches to measure GPCR internalisation have been developed, including post-internalisation labelling of residual surface receptor, TR-FRET measures of receptor internalisation[618], amongst others, and could be used in future to precisely quantify subtle differences in receptor trafficking.

As both SRB103His3 and SRB103Gln3 are matched in amino acid sequence apart from the third amino acid, it is considered unlikely that the peptides have different pharmacokinetic profiles. However, *in vitro*, it is known that electron-rich amino acids such as histidine are prone to photo-oxidation[619,620], which could take effect upon long stimulation times. Therefore, differences in peptide stability, which could explain increased cAMP efficacy after 16 hours of treatment, is explored in section 4.2.1.

SRB103Gln3 does show similar pharmacological signalling profiles at the GCGR compared to Ex-phe1 at the GLP-1R[276]. Both are partial agonists for $G\alpha_s$ recruitment acutely but increase in potency and efficacy for cAMP production after chronic stimulation. Reducing acute GCGR signalling could reduce acute hyperglycaemic events which are associated with glucagon signalling[621], but allow for prolonged insulin secretion and/or sensitivity which has been observed with chronic GCGR agonist administration[12,394,622]. Signalling at the GLP-1R, provided by the OXM backbone of the SRB103 compounds, will also aid in preventing GCGR-mediated acute hyperglycaemia. The interesting signalling properties and potential therapeutic benefits discussed of SRB103Gln3 make it interesting to take forward into further *in vivo* characterisation, which will be discussed in the following chapters.

4 Acute in vivo characterisations of biased dual GLP-1R/GCGR agonist

4.1 Introduction

Currently, candidate drugs must be tested for efficacy and toxicology in two species before exposure to humans. Whilst there are caveats to the translation of findings in non-human species, testing on animals in the field of diabetes has aided in discovering the very basis of glucose homeostasis to generating the next wave of anti-diabetic pharmaceuticals. In this chapter, the SRB103 peptides are taken into acute *in vivo* testing to uncover the therapeutic potential of a dual GLP-1R/GCGR agonist with reduced β-arrestin efficacy.

4.1.1 Current in vivo models used for diabetes and obesity research

Type 2 diabetes and obesity can be induced in animals in two ways: genetic manipulation or diet alteration. Currently, rodents make up the majority of animal species used in diabetes research due to the combination of small size, short life cycle, extensive genomic understanding and physiological similarities to humans[623]. Mice, in particular the C57BL/6 strain, represent the majority of animal testing cases[624]. C57BL/6 mice themselves have a variety of sub-strains, each with unique metabolic profiles which need to be considered. The 6J sub-strain, the most commonly used sub-strain from the Jackson Laboratory, are typically used in diabetes and obesity research as they are predisposed to developing insulin resistance and glucose intolerance in both a lean state and when fed on a high fat/high sugar diet[625]. The 6J sub-strain express a mutant *Nnt* gene which codes for the mitochondrial pump nicotinamide nucleotide transhydrogenase (NNT), which results in a reduction in glucose-stimulated insulin release compared to the 6N sub-strain[626-628]. Despite these sub-strain abnormalities, the extensive use of the 6J sub-strain means that they remain popular in current diabetes and obesity research. Indeed, key papers evaluating GLP-1R agonists and dual GLP-1R/GCGR agonists have predominantly used the 6J strain[276,442,456,464,502,629-632].

Whilst predisposed to obesity, further manipulation of the 6J sub-strain through diet or genetics is still used to induce a diabetes and/or obesity phenotype. Diet-induced obesity is usually preferred to manipulation of single genes, as the majority of human diabetes and obesity results from environmental factors and polygenic variation, with only a fraction attributable to monogenic factors[625,633,634]. Standard RM1 diet (Special

Diet Services, UK), used in the lean animals in this work, derives its total caloric energy from 7.5% fat, 17.5% protein and 75% carbohydrates. High fat diets typically switch carbohydrate-derived calories for fat-derived calories, resulting in a diet in which approximately 60% of caloric energy is derived from fat[635]. Another diet used is the high-fat/high-sucrose diet or "western diet", which some argue is more representative of the modern energy-rich diet consumed in the developed world[636]. The typical result of this is obesity, hyperlipidaemia, hyperinsulinaemia and impaired glucose tolerance, however there is the heterogeneity in diabetic phenotype between mice[637]. Mice have even been shown to be resistant to diet induced obesity due to an adaptive response elicited by microbiota sub-populations[638]. Despite this, diet-induced obese mice are commonly used to investigate the acute anti-hyperglycaemic abilities of GLP-1R and dual GLP-1R/GCGR agonists[276,442,502,520].

Genetic variants of diabetic rodents can be monogenic and polygenic. The major advantage of using genetic strains over diet-induced diabetes and obesity is the lack of need for time consuming and variably successful feeding regimens to induce the condition[639], although obesogenic diets are often used in combination with genetically susceptible strains to maximise the metabolic phenotype. Human T2DM and obesity have a predominantly polygenic origin, and therefore polygenic strains are more representative of the human condition[640-644]. However, monogenic mouse strains are also commonly used, partly for reasons of tradition and historical context, and partly as they display more extreme phenotypes which allow treatment effects to be more easily distinguished. One well-established example is the db/db mouse, which carries a mutation in the leptin receptor (*lepr*$^{-/-}$) (discussed in section 1.2.1), which displays a hyperphagic phenotype, resulting in obesity, hyperinsulinaemia, hyperglycaemia and dyslipidaemia, imitating the human condition[645,646]. To accentuate a diabetic phenotype, db/db mice can be bred in the C57BL/KsJ mouse strain, however these have a short life span of approximately 6 to 8 months[647]. Homozygous db/db mice are also infertile, therefore heterozygous mating has to be performed to generate a cohort of *lepr*$^{-/-}$ males. Therefore, the cost of generating a usable cohort is substantial. Another monogenic mouse line is the ob/ob mouse, which has homozygous knockout of the gene producing leptin itself. These mice present with similar phenotypes to db/db mice[648]. Despite this, the ob/ob mice have several disadvantages. Firstly, there is not complete ablation of β cell function or insulin signalling when ob/ob mice are

bred on a C57BL/6 background meaning the extent of type 2 diabetes phenotype is typically mild in comparison to the db/db mice[639]. More severe diabetes can be produced in ob/ob mice bred on a C57BL/KsJ background, however these mice suffer from early mortality[649]. Homozygous *lep*$^{-/-}$ rodents are also infertile[650]. Both of these lines can be also be fed HFD to induce a more extreme disease phenotype[651-653].

As mentioned, polygenic diabetic strains likely represent a closer resemblance to human type 2 diabetes compared to monogenic strains. They also develop less-extreme symptoms which may also more closely resemble the condition[640,642]. Examples of polygenic diabetic obese mouse strains include the TallyHo/Jng mouse, NoncNZO10/LtJ mouse and New Zealand Obese (NZO) mouse. NZO mice are hyperleptinaemic, resulting in obesity and concomitant hyperinsulinemia and β cell hypertrophy[654,655]. The other two are bred to develop diabetes meaning there is a greater yield of diabetic mice, however the NoncNZO10/LtJ mouse are non-obese and only develop typical type 2 diabetic pathophysiology[640]. Whilst they represent the human condition more closely, as they are polygenic it is not possible to produce littermate controls, and a strong male sex bias has been reported in many polygenic mouse strains[641]. NZO mice also display immune deficiencies, which can influence the pathophysiology of obesity and hence affect the validity of translating results into humans[656,657].

Rats are the second most commonly used rodent species used[658]. Due to a larger and more complex physiology, rats are preferred in some studies as the dissection and manipulation of tissues and organs is easier than in mice, and with a larger blood volume, more regular blood samplings can be done. Secondly, it has been shown that a rat's diabetic state can more readily adapt to environmental factors such as stress, social differences and toxins compared to mice, more reflective of the human state[659]. Rats were typically preferred to mice when investigating diabetic cardiomyopathy and cardiovascular pathophysiology due to the size and complexity of rat cardiovascular system (CVS), however mice are increasingly used due to advances in microsurgery and miniaturisation of surgical technologies[660,661]. There are disadvantages to choosing rats over mice when investigating T2DM and obesity. Intuitively, as rats are larger, the cost of maintaining rats is more than that of mice. There is also a greater understanding of the mouse strain and sub-strain genomes, and improved molecular

techniques allow for greater diversity of potential studies. Intriguingly, Ex4, a commonly used GLP-1R agonist, causes unexpected transient hyperglycaemia after administration, believed to be centrally mediated[662]. This is not seen in humans, and therefore raises concerns as to the validity of using rats as a model for GLP-1R activity.

Like mice, rat strains have been selectively bred to spontaneously induce diabetes. The Zucker Fatty (ZF) rat is a commonly used monogenic *lepr*$^{-/-}$ rat strain used in T2DM research. Like db/db mice, ZF rats show obesity and reduction in insulin sensitivity and glucose tolerance, but resting normoglycaemia[647]. Selective breeding of the ZF rat lead to a less obese, more diabetogenic strain (due to increased β cell apoptosis) called the Zucker Diabetic Fat (ZDF) rat[663,664]. Like the lepr-/- mice, ZDF rats are infertile, therefore carry many of the disadvantages that the db/db mice have, however there are efforts to generate ZDF strains with improved fertility to circumvent this[665]. Polygenic diabetic rat strains are also available, including the Otsuka Long-Evans Tokushima Fat (OLETF) and Goto Kakizaki (GK) rats. OLETF rats are mildly obese and the males display hyperglycaemia after approximately 18 weeks, arising from β cell necrosis[640,666]. GK rats, however, are non-obese and develop diabetes due to reduced β cell mass and function[667-669]. Therefore, depending on the specifics of the research question at hand, either of these strains provides a good model for type 2 diabetes. The hyperglycaemic and insulin deficient phenotype in rats can also be accentuated by feeding with HFD to induce a diabetic or obese phenotype[640,670].

Pharmacological means of inducing T2DM phenotypes are lacking. Currently, streptozotocin is the most commonly used drug used to induce diabetes, resulting in destruction of the β cell[671]. Whilst this does result in severe hyperglycaemia, due to a lack of insulin secretion, it likely does not represent the broad pathophysiology of obesity-induced T2DM, of which insulin secretion is initially increased, but insulin sensitivity is greatly reduced.

Larger mammals have also been used in diabetic research, such as cats[672], pigs[673-675], primates[676,677] and dogs[678,679]. Whilst the first three species show significant resemblance to human type 2 diabetes and therefore offer advantages over rodents in incretin therapy research, an FDA report showed dogs had adverse testicular reactions to lixisenatide dosing, suggesting their use in GLP-1R agonist testing is

limited[680]. The increase in cost and housing, along with ethical issues, also makes non-primary species less viable to use.

4.1.2 Transgenic mouse strains to investigate incretin biology

Generating and testing transgenic mouse lines has been a critical validation step for many mechanistic, physiological and pharmacological studies, allowing for ablation, overexpression and selective activation of key proteins, receptors and enzymes. For incretin biology, many GLP-1R and GCGR knockout models have been described that allow for whole-body or tissue-selective knockout of either receptor in a congenital or inducible manner.

The first example of GLP-1R knockout- mice was generated by manipulating the *Glp1r* gene, resulting in a null *Glp1r* gene[681]. Similarly, the first examples of mice lacking the GCGR were developed using a similar process of germline genetic manipulation resulting in a null gene[354,682]. The result is a complete lack of functional gene. Global, congenital knockout of genes can result in phenotypic alterations not witnessed in wild-type animals, which may partly result if the gene plays an important role during development, and partly due to pre-natal and post-natal physiological adaptions. For example, both GLP-1R and GCGR knockout lines result in hypersecretion of the cognate ligand[354,683], upregulated secretion of other incretins such as GIP[684], and morphological changes and hyperplasia of key endocrine tissues such as the islet[354,685].

In an attempt to reduce morphological and secretory alterations, Cre-Lox recombination can be used to generate tissue-specific or conditional transgenic mouse lines. Here, Cre-recombinase expression is driven by a specific promoter allowing manipulation of genes flanked by two LoxP sites. Depending on the orientation of the LoxP sites, it allows for gene activation, deletion, inversion or translocation[686]. The process of gene deletion, used in this project, is illustrated in Figure 4.1.

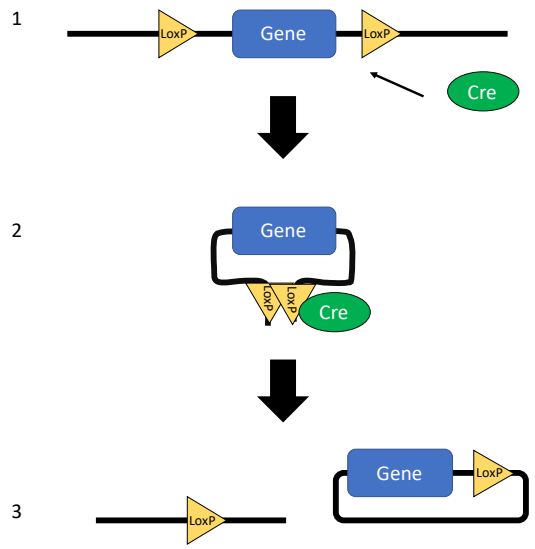

Figure 4.1 - Illustration of Cre-LoxP gene deletion

(1) LoxP sites (yellow) flank the gene of interest (blue) in the same orientation. (2) Upon tissue or treatment-specific expression of Cre recombinase (green), LoxP sites are joined by Cre recombinase which initiate recombination. (3) The gene segment is excised from the genome where the Cre was expressed, resulting in knockdown of the target gene.

The Cre-Lox recombination tool allows for tissue-specific gene deletion and has been widely used to produce whole-body and tissue-specific deletion of both the *Glp1r* and *Gcgr*. This approach allows the study of the effects of the endogenous ligand (or drugs targeting the receptors) in different tissues[686].

To minimise the impact that loss of any developmental function in germline or tissue-specific knockout models, inducible-Cre promoters can be used, in which Cre activity is only induced after treatment with a specific endogenous ligand. A Cre-recombinase under the control of a primer containing a fragment of the oestrogen receptor, meaning expression is controlled by administration of tamoxifen, is commonly used for this purpose[687]. In the case of GCGR, the adaptive responses to GCGR knockout are delayed compared to the complete ablation of the receptor itself, for up to 2 weeks[683]. It should be noted that due to tissue-related differences in exposure to tamoxifen and the variable capacity to synthesise Cre, 100% knockdown is rarely achieved by inducible models[688]. Moreover, not all cells will have the gene knocked out, regardless of the same expression of Cre[689,690].

Other methods exist for genetic manipulation of animals. Adeno-associated virus (AAV) is a widely used technique and has been used to knock down both receptors in specific regions/organs of the body[691]. Here, AAV have genetic constructs such as small interfering RNA (siRNA) or small hairpin RNA (shRNA) inserted into their genome which interferes with expression of the protein of interest. AAVs are a

particularly useful tool, as they display very low pathogenicity, eliciting a very mild immune response and have very low donor genomic uptake (around 0.1 - 0.5%)[692,693]. Different AAV forms also display variable tropism to different cell types, allowing for a degree of tissue-specificity to be achieved[694,695]. They also target both dividing and quiescent cells which expands the range of cells they can infect. Due to the small genomic size of AAVs, the genetic insert cannot exceed approximately 4-5kb, which limits its scope, however this is rarely a problem for siRNA or shRNA which is typically short in sequence[696].

Recent discovery of CRISPR-Cas9 technologies provide an exciting opportunity to selectively suppress, mutate or activate genes in the mouse embryo. The technology has been used to investigate genetic components of diabetes and obesity in mice[697,698], and zebrafish[699-701]. Whilst the technology is relatively straightforward (compared to previous gene editing approaches such as transcription activator-like effector nucleases (TALENs), and CRISPR mouse lines have been produced including those with GLP-1R deleted[702], CRISPR-Cas9 technology has been linked to genomic instability and off-target effects, as well as mosaicism arising from insufficient Cas9 cleavage at different cell cycle states[703,704].

4.1.3 Measuring glucose and insulin homeostasis in vivo

Dual agonists show exciting therapeutic potential to combat both diabetes and obesity. Assessment of their therapeutic efficacy requires robust and dynamic measurements of glucose and insulin responses.

Glucose tolerance tests (GTTs) are the first line of *in vivo* test for assessment of glycaemic effects of candidate drugs due to the ease to which they can be done. GTTs can be performed in a number of different ways depending on the research question they seek to address. Firstly, GTTs can be performed by oral gavage of glucose, termed an oral GTT (OGTT). As oral glucose instigates the release of endogenous incretins (discussed in section 1.1.1), it is a good measure of the body's own ability to control glucose excursions under physiological conditions[705]. As a major GLP-1R action is to slow gastric emptying, the overall glycaemic response to oral glucose after GLP-1R agonist administration results primarily from a combination of delayed

glucose delivery to the circulation and direct β cell stimulation. To better isolate the effects on β cells, glucose can also be administered intravenously (IVGTT) or, more practically, into the highly vascularised peritoneal cavity (IPGTT), where glucose absorption is nearly as rapid as an IVGTT[706]. Other routes of glucose administration are described, such as intragastric (IGGTT), which provides a separate means of inducing endogenous incretin secretion[707], however this technique is more technically challenging and usually requires larger animals to cannulate the gastro-intestinal tract. Therefore, IPGTT is the primary approach used in the assessment of incretin biology, with OGTT also frequently used as a more "complete" assessment of glycaemic physiology.

Another consideration to make is the dosing of glucose, especially with regards to lean *versus* obese mice. Glucose can be dosed based on total body weight or lean body mass. In a lean C57BL/6J mouse model, these two are quite similar, as a lean adult male has a fat-to-body weight percentage of approximately 25%[708,709]. However, DIO C57BL/6J mice can reach a fat-to-total body weight percentage of 40% without a great change in lean mass[709]. As lean mass is more closely aligned between lean and obese rodents, and skeletal muscle is the primary tissue for glucose uptake, it has been argued that glucose dosing should be carried out using lean mass weight to match for glucose uptake ability[710]. Furthermore, genetically-induced obese mice, such as db/db and ob/ob mice show a disproportional increase in total weight gain compared to blood volume[711]. If the blood volume of DIO mice is approximately similar to lean comparators, then dosing based on total body weight may overestimate the glucose intolerance of DIO mice[710]. However, in experiments measuring the difference in glucose dosing based on total or lean body mass between lean and DIO mice, there is little difference in results and the trend of greater intolerance in DIO mice was observed in both studies[710].

Whilst technically easy to perform, glucose tolerance tests in general cannot distinguish between reduced β cell function and increased insulin resistance. Insulin tolerance tests (ITTs), where insulin is injected in a fashion identical to GTTs, is used to evaluate the peripheral response to an increase in circulating insulin, e.g. a reduction in glycaemia. As insulin is injected at basal fasting glucose levels, there is a risk of hypoglycaemia with ITTs. This means optimisation of injected insulin

concentrations is required depending on the model used and experiment performed. The absolute or percentage decrease of basal glycaemia is a readout of insulin sensitivity and can be used to evaluate the ability of a treatment to improve insulin sensitivity providing the baselines are similar. This technique correlates well with other techniques used to measure insulin sensitivity, such as a euglycaemic hyperinsulinaemic clamp[712]. In mice, ITTs have been widely used to demonstrate the ability of GLP-1R agonists and GCGR agonists, to improve insulin sensitivity[394,502,713,714].

Dynamic measuring of insulin sensitivity, however, remains as the "gold standard" of measuring insulin secretion and sensitivity[715]. Glucose clamps, where the jugular vein and carotid artery are catheterised to allow for infusion of glucose and insulin, illustrate insulin secretion and sensitivity. Two main forms preside, each offering slightly different measurements. In a hyperglycaemic clamp, high concentration glucose is continuously infused into the animal to induce hyperglycaemia, and the rate of glucose infusion subsequently varies throughout to maintain a steady circulating hyperglycaemic state. This measures the subject's ability to produce and secrete insulin, as reflected in the amount of glucose needed to maintain hyperglycaemia. In humans, regular blood sampling for insulin measurements is possible, although this is limited in small rodents due to the available circulating blood volume. In contrast, hyperinsulinaemic-euglycaemic clamps use constant hyperinsulinaemic infusion and a variable glucose infusion to maintain euglycaemia. Whilst the hyperglycaemic clamp measures endogenous insulin production, hyperinsulinaemic-euglycaemic clamps measure the ability of the tissue to use the insulin, i.e. insulin sensitivity[716,717]. These clamping methods are considered the "gold standard" measures, respectively, of insulin secretion and sensitivity assessments. They can be technically challenging, time-consuming and require more expertise than do GTTs and ITTs, therefore GTTs are more routinely used as a basic readout for glucose control for screening purposes.

Hyperglycaemic clamps can be used to infer insulin secretion but as an indirect readout only. For this reason, assays quantifying blood insulin concentrations are available and widely used. As described in section 3.1.3, ELISAs can provide very accurate measurements of substrate[718]. For an insulin ELISA, the sensitivity of assays can be as low as 1.1pmol/L[719]. The working volume of plasma needed for the assay

is also as low as 5µl, which means it is not particularly invasive to the animal and can be done multiple times[718]. Serum insulin levels can also be measured with an RIA[720], and advantages and disadvantages of these are discussed in section 3.1.3. Circulating insulin concentrations can be used alone as a marker of the efficacy of an anti-diabetic treatment, however they are routinely used in tandem with other readouts to confirm how the insulin in being used.

Fasting insulin concentrations can also be used with fasting glucose levels as part of the homeostatic model assessment (HOMA), which is a quantifiable calculation of either insulin resistance or β cell function[721]. Two forms of HOMA calculation are used: HOMA-β (measuring β cell function) and HOMA-IR (measuring insulin resistance), of which HOMA-IR is used much more regularly that HOMA-β[722]. HOMA models are used to illustrate the relationship between the resting glucose level and how much insulin is needed to produce that basal glycaemic level. Despite being used to approximate animal insulin resistance, the model is only validated in humans, and it is debated whether the formulae are applicable to other species[722].

4.1.4 Measuring food intake in mice

Obesity is due to an increased energy intake relative to energy requirements. Therefore, measuring food intake is a key component in investigating obesity.

Depending on the specific question, food intake studies can be performed acutely (less than a day) or chronically. Acute studies are normally used to investigate preliminary satiety effect and, if possible, are performed nocturnally as mice predominantly eat in the dark phase[723]. If the drug is suspected to induce effects in energy expenditure, pair-feeding approaches can be performed, where vehicle-treated mice are given a matched daily food weight. This would eliminate the effect of excess food intake observed in obese mice. As obesity and type 2 diabetes are multifactorial processes, pair feeding experiments omit the role of overall food intake on important processes such as energy expenditure[724-726] and thermoregulatory thermogenesis[727] in obese mice where total food intake could be a confounding factor. Isocaloric pair-feeding studies have also been used to investigate the individual roles of components of food such as fat, carbohydrates and protein in weight management[95,538,637,638].

GLP-1R agonists are associated with reducing food intake, however, one of their side effects which reduces usability and patient compliance is induction of nausea[326]. Unlike humans, rodents do not have a vomit reflex, but they do exhibit behaviours such as reduced eating and pica (eating non-nutritive substances such as bedding or kaolin clay)[728]. Screening of GLP-1R agonists routinely includes measurements of food intake to assess their anorectic properties. It has been shown that a biased Ex4 analogue, which caused improved glucose tolerance over 8 hours compared to Ex4, displayed no change in food intake, whereas Ex4 inhibited food intake[276]. There is controversy as to whether glucagon increases or decreases food intake, whether these effects are a primary or secondary cause of GCGR agonism, and how this impacts glucagon's role in energy expenditure. Interestingly, OXM reduces food intake in rodents and in mice, the anorectic effect of OXM was ablated by injection of GLP-1R antagonist exendin(9-39) into the arcuate nucleus of the hypothalamus, widely considered as an integral feeding centre[429]. In a separate study where glucagon receptor activity was ablated in OXM, food intake was not affected[729]. This suggests the GLP-1R element of OXM and GLP-1R/GCGR dual agonists may be driving food intake reductions centrally.

4.1.5 Aims

The aim of this following chapter is to:

1) Determine how SRB103His3 and SRB103Gln3 affect metabolic parameters *in vivo*, including glycaemic control, insulin secretion and food intake.
2) Examine therapeutic changes, if any, between lean mice and diet-induced obese (DIO) mice.
3) Establish whether the effects of SRB103His3 and SRB103Gln3 are affected by disruption of GCGR signalling.
4) Confirm the role of β-arrestin signalling in the *in vivo* pharmacology of the separate SRB103 peptides.

4.2 Results

4.2.1 Acute anti-hyperglycaemic effect of SRB103 peptides in lean mice

Sustained insulinotropic properties of GLP-1RAs can be enhanced through selective signalling to enhance cAMP signalling[276,502,504]. Lean C57BL/6J male mice were therefore tested for their ability to tolerate a glucose excursion after i.p. glucose administration after acute, 4 hours or 8 hours treatment with the SRB103 peptides or vehicle. Mice were fasted 4 hours before i.p. peptide administration for acute and 4-hour studies, and one hour before 8-hour studies to reduce the impact of extended fasting on glucose readings.

After acute peptide administration (Figure 4.2A, left panel), there was no significant difference between either SRB103His3 or Gln3 in their anti-hyperglycaemic abilities, as calculated through AUC analysis, as measured by two-way ANOVA of the AUC ($P>0.05$) (Figure 4.2B). Whilst no difference is seen between the two SRB103 peptides, SRB103Gln3 produces an improved anti-hyperglycaemic response compared to vehicle ($P<0.001$), whereas SRB103His3 displays no difference with vehicle ($P>0.05$), suggesting SRB103Gln3 is marginally more effective at correcting hyperglycaemia acutely. However, after 4 hours of peptide treatment, SRB103Gln3 produces a statistically significant improvement in glycaemic control compared to SRB103His3 ($P<0.05$), as well as a decrease in pre-glucose baseline (4.3 ± 0.2mM *versus* 5.8 ± 0.3mM; $P<0.001$) (Figure 4.2A, middle panel). The divergence in total IPGTT AUC between the two SRB103 peptides is further increased at 8 hours ($P<0.0001$) (Figure 4.2B).

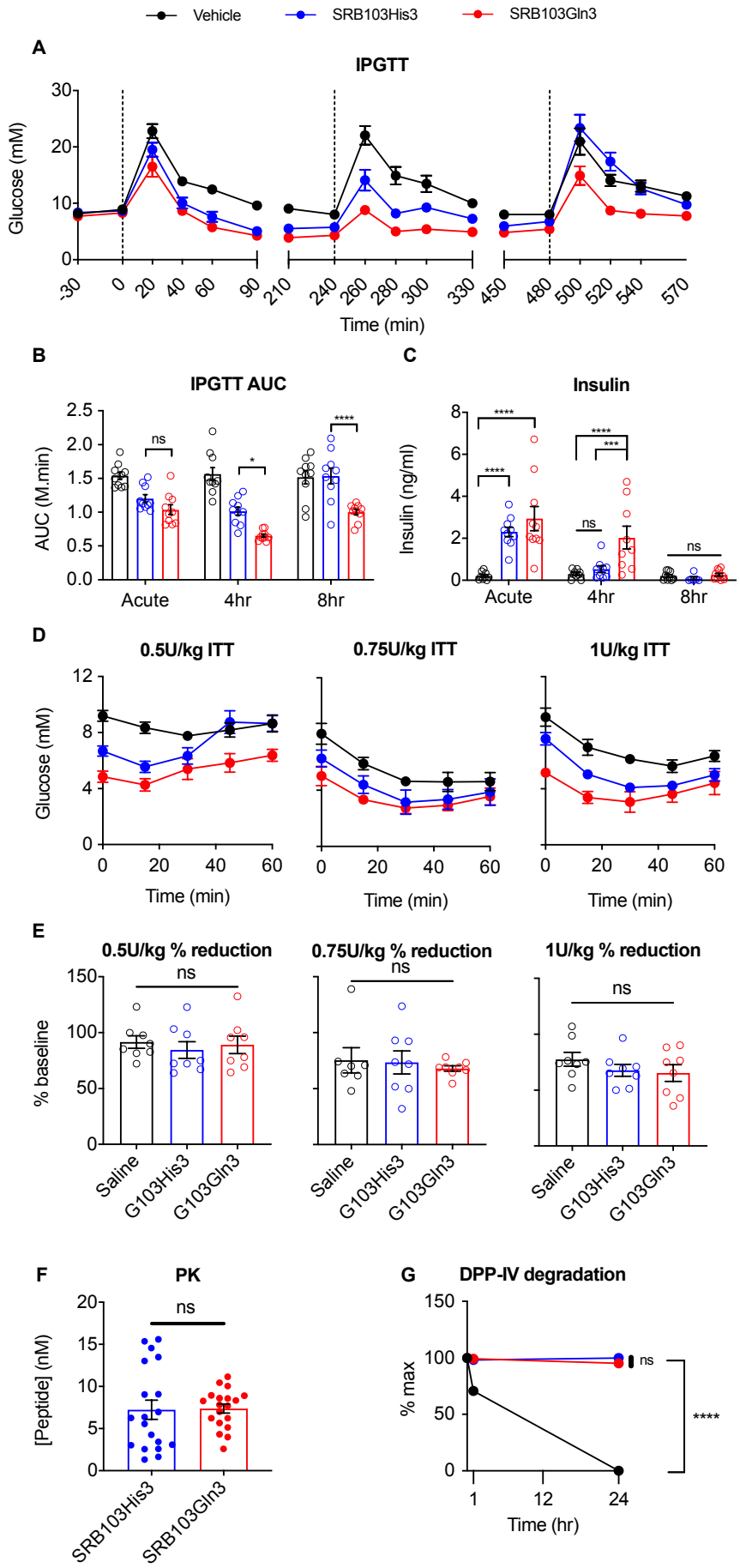
Vehicle
SRB103His3
SRB103Gln3
A
IPGTT
Glucose (mM)
Time (min)
B
IPGTT AUC
AUC (M.min)
Acute
4hr
8hr
C
Insulin
Insulin (ng/ml)
D
0.5U/kg ITT
0.75U/kg ITT
1U/kg ITT
Glucose (mM)
Time (min)
E
0.5U/kg % reduction
0.75U/kg % reduction
1U/kg % reduction
% baseline
Saline
G103His3
G103Gln3
F
PK
[Peptide] (nM)
SRB103His3
SRB103Gln3
G
DPP-IV degradation
% max
Time (hr)

Figure 4.2 - Effects of SRB103 peptides on glycaemic control in lean mice

Lean C57BL/6J mice (3-6 months) were fed on standard chow. (A) IPGTT results in mice dosed either acutely, 4 hours post or 8 hours post i.p. injection of 10nmol/kg SRB103Gln3 (red), SRB103His3 (blue) or vehicle (black). Mice were fasted 4 hours prior to acute and 4-hour testing and 1 hour before 8-hour testing. Pre-glucose bolus glucose levels were taken 30 minutes and immediately before 2g/kg glucose (in 0.9% NaCl solution) was administered i.p. (dotted line) and blood glucose measured was measured periodically afterwards (n=10). (B) Area under curve (AUC) of (A) calculated as M.min. (C) Insulin secretion after acute, 4-hour or 8-hour SRB103 peptide or vehicle treatment. At each treatment time point indicated, 2g/kg glucose was administered i.p., and blood samples taken 10 minutes after by venesection. Insulin measured by HTRF (n=9-10) (D) Insulin tolerance test (ITT) in fasted mice treated for 4 hours with 10nmol/kg SRB103 peptide or vehicle. Insulin (in 0.9% NaCl solution) was administered i.p. with doses of 0.5U/kg (left), 0.75U/kg (centre) or 1U/kg (right) at time 0 (n=8) (E) Percent reduction in initial blood glucose, measured as [mean average blood glucose t_{15}/mean average blood glucose t_0] (n=8). (F) 4 hour circulating levels of 0.5mg/kg SRB103His3 or SRB103Gln3 in mice as measured by radioimmunoassay (n=20). (G) DPP-IV degradation rate of 16.7μM SRB103 compounds (red and blue) and GLP-1 (black). Incubation at 37°C as indicated and reaction terminated with 0.05% trifluoracetic acid. Data given relative to baseline (n=3). Data presented as mean ± SEM. Statistical significance calculated using one-way (treatment) or two-way (treatment and time) ANOVA with Tukey post hoc test (B, E, G), mixed-effects test with Tukey post hoc test (C) or student t-test (F). Error bars show SEM. **** P<0.0001; *** P<0.001; * P<0.05; ns - not significant (P>0.05).

Interestingly, while SRB103His3 reduces the induced hyperglycaemia by the IPGTT 4 hours after the injection compared to vehicle, the effect is lost if injected 8 hours prior to the IPGTT, suggesting it has a shorter efficacy (P>0.05). A similar trend was replicated with four further His3/Gln3 compound pairs (Table 4.1). Here, two measurements of pre-glucose and 30-minute measurements were used as a surrogate for total glucose tolerance. Despite all four Gln3 peptides showing a trend of increased glucose tolerance at 4 hours post-injection, only one His3/Gln3 pair showed significant differences due to the variation in the data, improved GLP-1R efficacy of SRB103Gln3 compared to the other Gln3 compounds used and smaller n numbers.

		His1	Gln1		His2	Gln2		His3	Gln3		His4	Gln4	
Acute	**Pre**	8.4 ± 0.4	8.3 ± 0.1	ns	7.4 ± 0.5	8.1 ± 0.2	ns	7.6 ± 0.4	7.4 ± 0.2	ns	8.1 ± 0.3	8.6 ± 0.4	ns
	30m	14.0 ± 2.0	13.7 ± 1.6	ns	13.6 ± 0.8	13.1 ± 0.8	ns	12.9 ± 1.5	12.5 ± 1.0	ns	15.9 ± 1.1	18.7 ± 1.5	ns
4h	**Pre**	5.8 ± 0.6	5.1 ± 0.3	ns	5.1 ± 0.6	4.6 ± 0.6	ns	5.2 ± 0.3	5.1 ± 0.5	ns	6.9 ± 0.9	5.1 ± 0.6	ns
	30m	12.2 ± 1.9	9.8 ± 1.6	ns	13.5 ± 3.5	10.8 ± 1.5	ns	14.5 ± 1.4	11.1 ± 1.5	ns	18.9 ± 2.1	13.2 ± 1.7	**

Table 4.1 - Anti-hyperglycaemic effects of four other AIB2Gln3/His3 peptide pairs

Mice were fasted 4 hours before the study. Glucose measurements taken before (Pre) and 30 minutes after (30m) 2g/kg glucose i.p. (n=4-8). Statistical analysis performed by two-way ANOVA with Tukey post hoc test. Data presented as mean ± SEM. ** P<0.01; ns – not significant (P>0.05).

Having confirmed SRB103Gln3 produced greater anti-hyperglycaemic effects than SRB103His3, potential mechanisms were investigated further. The two presiding theories here for improved anti-hyperglycaemia are:

1) Increased insulin secretion
2) Increased insulin sensitivity

To investigate the former, SRB103 peptides or vehicle were administered to lean mice. After acute, 4 hours or 8 hours of treatment, 2g/kg glucose was injected and ten minutes later blood samples were taken to measure plasma insulin. Both SRB103 peptides induced a robust increase in insulin secretion when administered acutely (Figure 4.2C). However, after 4 hours of treatment, only SRB103Gln3 caused a significant four-fold increase in insulin release compared to SRB103His3 (two-way ANOVA; $P<0.001$). Interestingly, by 8 hours neither SRB103 peptides elicited a significant insulin release compared to vehicle ($P>0.05$). Cumulatively, these data suggest that SRB103Gln3 elicits its improved anti-hyperglycaemic effect through increased insulin secretion at 4 hours after treatment.

As GCGR agonism has been paradoxically linked to improvements in insulin sensitivity[394], effects on insulin sensitivity were also investigated by performing ITTs to observe the response of mice to both SRB103 peptides 4 hours after administration (Figure 4.2D). This was the time when the greatest anti-hyperglycaemic effect was observed. Using three separate insulin doses (0.5U/kg – 1U/kg) to ensure maximal insulin effects, insulin sensitivity was calculated by the percentage reduction in blood glucose at the fifteen-minute timepoint compared to initial insulin levels (Figure 4.2E). This approach was required as the “baseline” glycaemia between treatment groups were different. Using this method, no difference in insulin sensitivity was observed between agonists or vehicle (one-way ANOVA; $P>0.05$), suggesting prolonged SRB103Gln3 administration does not oppose hyperglycaemia through increased insulin sensitivity.

A further potential reason for the improved anti-hyperglycaemic phenotype observed is differences in pharmacokinetics (PK), as incretins are most sensitive to DPP-IV degradation at positions 1 and 2 of the molecule[730,731], close to the site of amino acid substitutions. To rule out differences in PK or DPP-IV sensitivity two approaches were taken. Firstly, C57BL/6J mice were injected via the i.p. route with SRB103 peptide and plasma samples taken 4 hours after. Plasma concentration of SRB103 peptide was confirmed using an RIA. No difference in plasma peptide concentration was observed

at 4 hours (student t-test; P>0.05) (Figure 4.2F). Further confirmation was conducted by incubating the SRB103 peptides or native human GLP-1 for up to 24 hours in buffer containing human DPP-IV. Samples were then analysed using high performance liquid chromatography (HPLC). Here, human GLP-1 showed full DPP-IV degradation by 24 hours, while SRB103 peptides showed statistically similar, negligible DPP-IV degradation (one-way ANOVA; P>0.05) (Figure 4.2G).

These data support the hypothesis that SRB103Gln3 elicits greater anti-hyperglycaemia compared to SRB103His3 due to distinct cAMP-selective signalling at one or both receptors, resulting in greater insulin secretion.

4.2.2 Acute anti-hyperglycaemic effects of SRB103 peptides in DIO mice

Having observed an anti-hyperglycaemic effect of SRB103Gln3 in lean mice up to 8 hours after administration, C57BL/6J mice were fed on HFD for 3 months and IPGTTs performed as previously described to confirm the effects in a pathological model of diet-induced obesity (DIO).

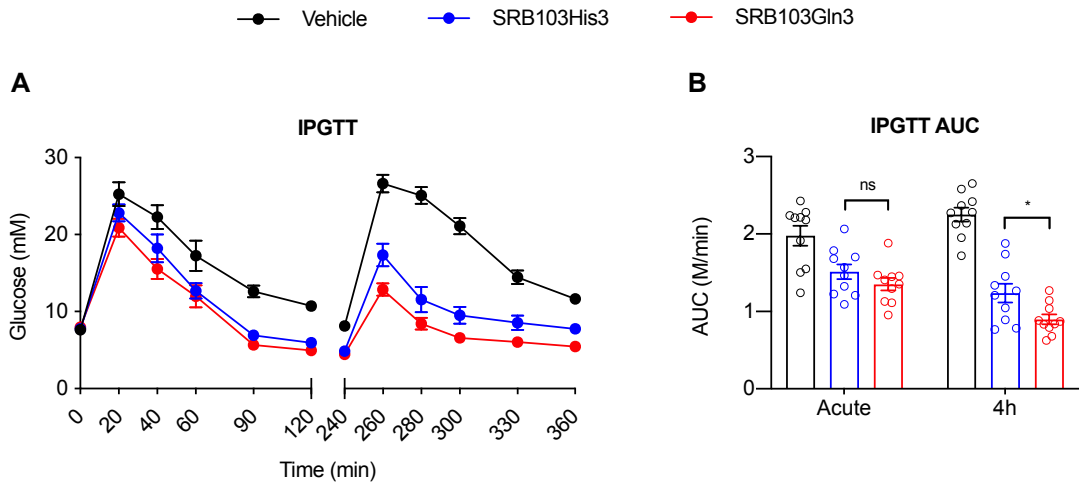

Figure 4.3 - Anti-hyperglycaemic effect of SRB103 peptides in DIO mice

Mice (6-9 months) were fed on 60% kcal high fat diet for at least 3 months before metabolic testing. (A) IPGTT results in DIO mice measuring the acute (left) or 4-hour (right) effect of SRB103His3 (blue), SRB103Gln3 (red) or vehicle (black). Mice were fasted 4 hours before the study. Peptide administered i.p. with 2g/kg glucose (in NaCl solution) for acute study or 4 hours before 2g/kg glucose administration (n=10). (B) Area under the curve for (A). Data expressed as mean ± SEM. Statistical analysis performed using two-way ANOVA with Tukey post hoc test. * P<0.05; ns – not significant (P>0.05).

Like for the lean mice, DIO mice were fasted for 4 hours before the test and injected with SRB103 peptide or vehicle either acutely or 4 hours before 2g/kg glucose administration. 8-hour treatment timepoints were omitted as there was a greater divergence in effect at earlier timepoints. DIO mice were not tested further for insulin resistance and conformation of diabetes, but the additional weight gain (approximately 14g on average compared to lean mice) and higher glycaemic values at 40- and 60-minute time points were convincing evidence of insulin insensitivity and progression towards a diabetes-like phenotype. As observed with the lean mice, no difference in the anti-hyperglycaemic effect was observed between SRB103Gln3 and SRB103His3 after acute peptide administration (Two-way ANOVA; $P>0.05$) (Figure 4.3). Interestingly, DIO mice displayed similar baseline glycaemic levels to lean mice, however this is likely an artefact of stress relating to the procedure. After 4 hours of peptide treatment, SRB103Gln3 produced an improved anti-hyperglycaemic effect *versus* SRB103His3 as observed in the lean mice ($P<0.05$). This corroborated earlier findings of improved anti-hyperglycaemia by SRB103Gln3 treatment in a DIO model, further implicating the unique signalling profile of SRB103Gln3 as a potential treatment of type 2 diabetes.

4.2.3 Pharmacological approaches to delineate anti-hyperglycaemic mechanisms

SRB103Gln3 shows a prolonged anti-hyperglycaemic ability not observed with SRB103His3. This effect is not related to pharmacokinetic differences between the peptides, but potentially reduced β-arrestin efficacy at either the GCGR (Figure 3.3) or the GLP-1R (Figure 3.4) respectively. In an attempt to delineate which receptor is responsible for the phenotype shown in the IPGTTs described above, acylated antagonists for GLP-1R and GCGR were designed based on widely used exendin9-39 (termed Acyl-Ex9) and DesHis1-[Glu9]-glucagon(1-29) amide (termed Acyl-DHG) respectively[732,733] (see Appendix).

Acyl-Ex9 was tested *in vitro* to ensure it acted as a competitive antagonist at the GLP-1R, and showed it was able to fully antagonise 1nM GLP-1 (Figure 4.4A). Having shown it could compete with GLP-1 *in vitro*, a 50nmol/kg Acyl-Ex9 dose was used for its ability to antagonise GLP-1-mediated anti-hyperglycaemia in an IPGTT (Figure

4.4B, left panel). Mice were pre-administered i.p. either Acyl-Ex9 or vehicle, followed 30 minutes later by 2g/kg i.p. on glucose with or without 0.24nmol/kg Ex4. There was a partial increase in AUC between Ex4-treated mice administered with Acyl-Ex9 compared to vehicle (One-way ANOVA; $P<0.05$) suggesting a minor reduction in Ex4 anti-hyperglycaemic effect (Figure 4.4B, right panel). The magnitude of the Ex4-induced reduction in glycaemia was similar with or without prior administration of Acyl-Ex9, suggesting that Acyl-Ex9 is not sufficient to unambiguously distinguish the GLP-1R-mediated effects of both SRB103 peptides.

Acyl-DHG was also tested for its ability to antagonise GCGR-mediated cAMP signalling *in vitro* (figure 4.4C). However, increasing concentrations of acyl-DHG did not reduce the cAMP increase mediated by 1nM glucagon. Indeed, at high doses, Acyl-DHG acted as an agonist, thereby limiting its ability to act as a competitive antagonist. Moreover, *in vivo*, Acyl-DHG (50nmol/kg) was unable to inhibit glucagon-induced hyperglycaemia in a glucagon challenge test ($P>0.05$) (Figure 4.4D) and was therefore not taken forward.

Therefore, a second small molecule reported as an orally available GCGR antagonist, L-168,049, was investigated as an alternative[734]. L-168-049 was a suitable GCGR antagonist in vitro as it fully antagonised 1nM glucagon (Figure 4.4E). To investigate L-168,049 *in vivo*, mice were initially orally gavaged (as recommended by the manufacturers) with vehicle or antagonist 30 minutes prior to 10nmol/kg glucagon or vehicle i.p. injection. Blood glucose levels were observed 15- and 30-minutes post-glucagon to determine if glucagon-induced hyperglycaemia was evident, then 2g/kg glucose administered i.p. and blood glucose monitored in order to observe any potential effect of glucagon-induced improvements in glucose tolerance[394]. In fact, L-168,049 did not block glucagon-induced hyperglycaemia, and L-168,049 alone resulted in increased total glycaemia alone *versus* saline treatment, as shown by total AUC ($P<0.05$) (Figure 4.4F). Also, contrary to finding from *Kim et al.*, glucagon alone didn't appear to display any anti-hyperglycaemic properties. In conclusion, it appeared that L-168-049 did not show sufficient GCGR antagonist properties to merit further use.

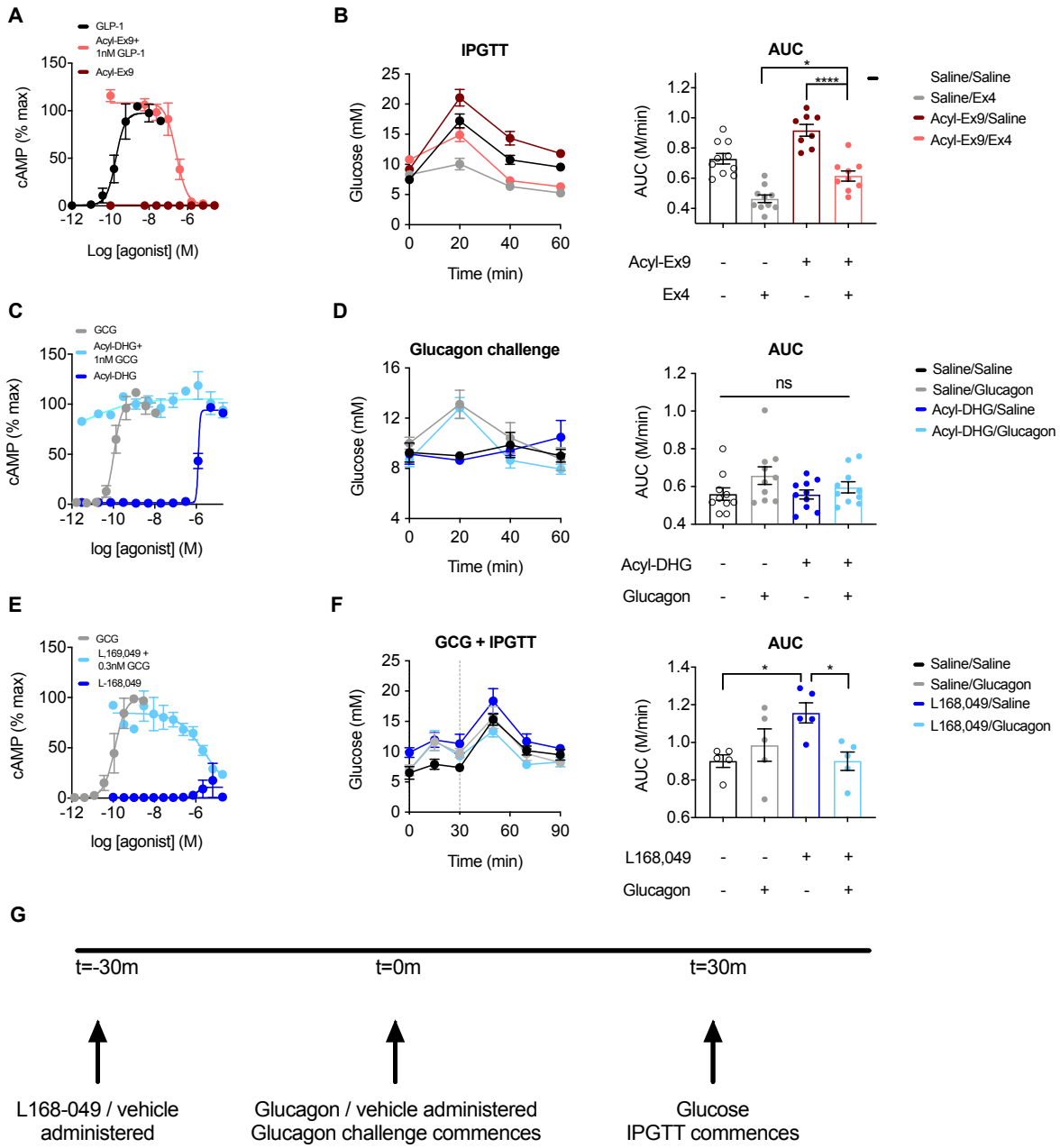

Figure 4.4 - Effect of acylated and oral GLP-1R and GCGR antagonists *in vitro* and *in vivo*

(A) Dose response of GLP-1 (black), Acyl-Ex9 (maroon), and Acyl-Ex9 + 1nM GLP-1 (pink) in DiscoverX CHO-hGLP-1R cells. Cells stimulated at 37°C for 30 minutes. Results normalised to GLP-1 E_{max}. (B) IPGTT in lean mice pre-treated for 30 minutes with 50nmol/kg Acyl-Ex9 (maroon, pink) or vehicle (black, grey). At t=0, i.p. glucose was administered containing either 0.24nmol/kg exendin-4 (grey, pink) or vehicle (black, maroon). (right) AUC of IPGTT given in M.min (n=8-10). (C) As for (A) but with Acyl-DHG and glucagon in DiscoverX CHO-hGCGR cells. (D) As for (B) but 20nmol/kg human glucagon administered or vehicle instead of 2g/kg glucose (n=10). (E) As for (C) but L168-049 used as the antagonist. (F) lean mice were dosed with 50nmol/kg L168-049 (light blue, dark blue) or vehicle (grey, black) then given an i.p. 25nmol/kg glucagon challenge (grey, light blue) or vehicle (black, dark blue) at t=0min. At t=30min all mice received 2g/kg i.p. glucose and an IPGTT preceded as before(n=5). (G) Schematic of glucagon challenge/IPGTT described in (F). Data presented as mean ± SEM. Significance calculated using one-way ANOVA with Tukey post hoc test. **** P<0.0001; * P<0.05; ns – not significant (P>0.05).

Cumulatively, these data suggest even if these GLP-1R and GCGR antagonists showed promising results *in vitro*, this does not translate to *in vivo* antagonistic efficacy. Therefore, another approach was sought to dissect the roles of these two receptors in the reduction of glycaemia observed after treatment with SRB103Gln3.

The second method trialled was to identify peptide sequences based on SRB103Gln3 and SRB103His3 which maintained characteristic biased signalling patterns at one receptor, whilst being inactive at the second receptor, termed “receptor-selective” peptides. Candidates were first selected according to historical cAMP signalling data generated from the in-house dual agonist database (Figure 4.5A). Two amino acid substitutions of interest were identified: serine at position 18 (Ser18) of the molecule, which increased cAMP signalling to the GLP-1R 35-fold (Figure 4.5A, top panel) and tyrosine at position 1 (Tyr1) which caused a 500-fold selectivity toward the GCGR (Figure 4.5A, bottom panel). SRB103 compounds were therefore synthesised with either Tyr1 or Ser18, in addition to the previously studied bias-inducing substitutions of His3 or Gln3 (see Appendix).

cAMP signalling responses of putative receptor-selective SRB103 peptides were then measured in DiscoverX CHO-K1 cells, with the aim of demonstrating that the introduction of Gln3 into Ser18 and Tyr1 peptides maintains reduced GCGR-specific β-arrestin recruitment without affecting other signalling properties. However, at the GLP-1R, Gln3Tyr1 was approximately 20-fold times more potent than His3Tyr1 for cAMP production (Figure 4.6B, top panel), and His3Tyr1 induced no β-arrestin recruitment at all whilst Gln3Tyr1 elicited weak β-arrestin recruitment, thus failing to replicate the negligible impact of the Gln3/His3 switch in the context of the native SRB103 peptide sequence (Figure 4.6B, bottom panel). Moreover, at the GCGR, whilst His3Ser18 exhibited higher β-arrestin efficacy than Gln3Ser18 (as intended), His3Ser18 was also approximately 18 times more potent for cAMP than Gln3Ser18 (Figure 4.6C, top panel). As assessment of the contribution of the specific reduction in GCGR β-arrestin efficacy to responses *in vivo* requires cAMP potency at both the GCGR and GLP-1R to be equivalent, the differences in potencies between the two Ser18 peptides at the GCGR and the two Tyr1 peptides observed at the GLP-1R meant this strategy was not appropriate as a method of delineating receptor contributions to the *in vivo* phenotypes.

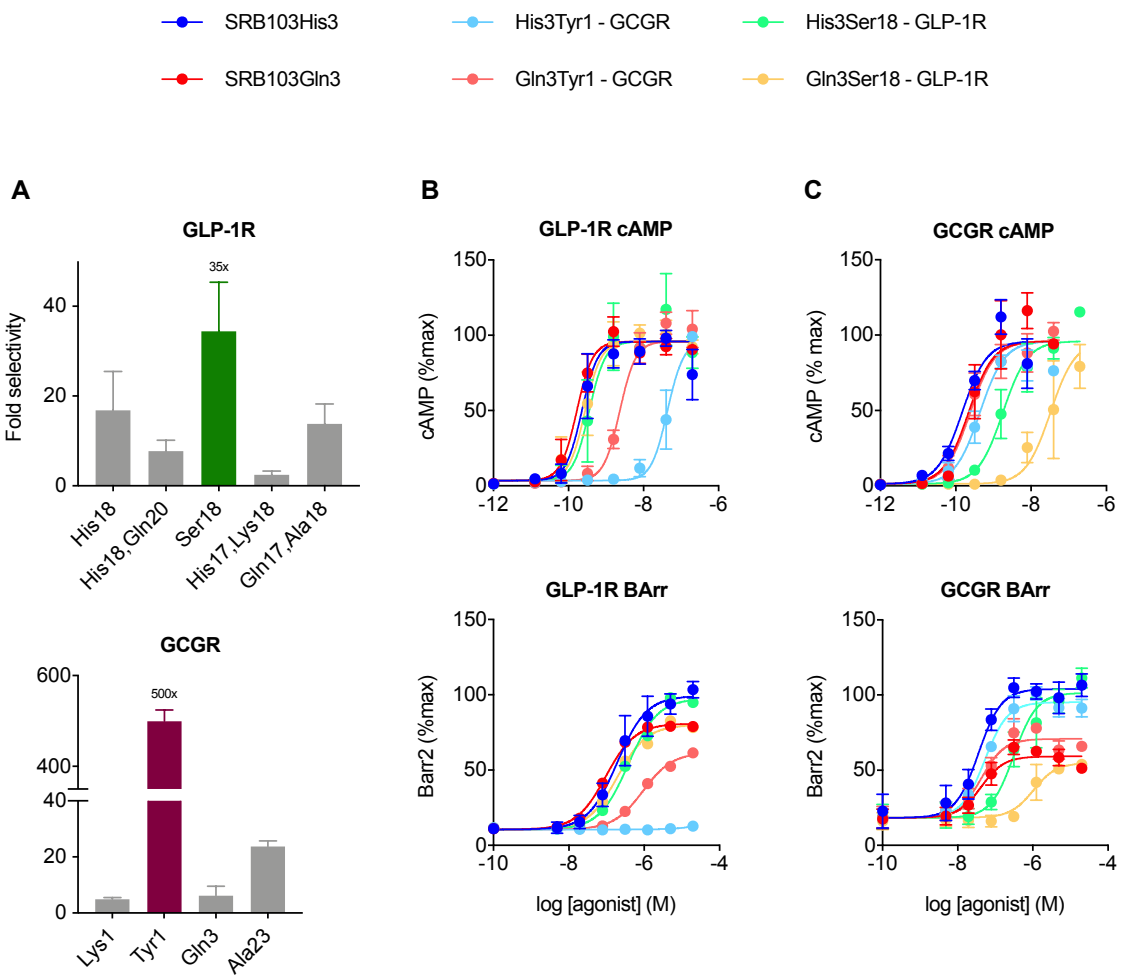

Figure 4.5 - Identification and validation of receptor-selective dual GLP-1R/GCGR agonists

(A) Selectivity for peptide with serine at position 18 (Ser18, green) to the GLP-1R (top) and peptide with tyrosine at position 1 (Tyr1, purple) to the GCGR (bottom). Dose response of peptides with amino acid substitutions listed in DiscoverX CHO-K1 hGLP-1R and hGCGR cells. Cells treated for 30 minutes at 37°C. Selectivity was calculated as [mean pEC_{50} favoured receptor/mean pEC_{50} second receptor]. (B) Dose-response of receptor selective SRB103 peptides in DiscoverX CHO-K1 hGLP-1R measuring cAMP (top) or β-arrestin (bottom). Peptides used were SRB103His3 (blue), SRB103Gln3 (red), SRB103His3Tyr1 (teal), SRB103Gln3Tyr1 (pink), SRB103His3Ser18 (green) and SRB103Gln3Ser18 (yellow). Cells were treated for 30 minutes at 37°C. Data was normalised to SRB103His3 E_{max} (n=3). (C) as for (B) but using DiscoverX CHO-K1 hGCGR cells. Data presented as mean ± SEM.

4.2.4 Acute anti-hyperglycaemic effects of SRB103 peptides in transgenic mice

As antagonists and receptor-selective peptides could not be used *in vivo* to define which receptor was responsible for the improvement in glycaemia observed in SRB103Gln3-treated mice, the next strategy was to produce receptor knockout mice. As initial data suggested SRB103Gln3 may be biased toward cAMP production at the GCGR compared to SRB103His3, mice with the GCGR knocked down were used to

evaluate the glycaemic response to an IPGTT after SRB103 peptide administration. Here, mice with the *Gcgr*$^{flox/flox}$ gene were crossed with either *Actb*-*Cre-ERT2* mice to produce a global tamoxifen-inducible GCGR knock out line (*Actb*-*Cre-ERT2*:*Gcgr*$^{fl/fl}$ or *Gcgr*$^{-/-}$), similar to that previously described[683] (here the *Actb* promoter is used rather than *Rosa26*), or *Alb*-*Cre* (under the *Albumin* promoter) to produce constitutive hepatocyte-specific GCGR knock out mice (*Alb*-*Cre*:*Gcgr*$^{fl/fl}$ or *Gcgr*$^{hep-/-}$)[735]. See Figure 4.6 for a schematic of the generation and use of transgenic mice.

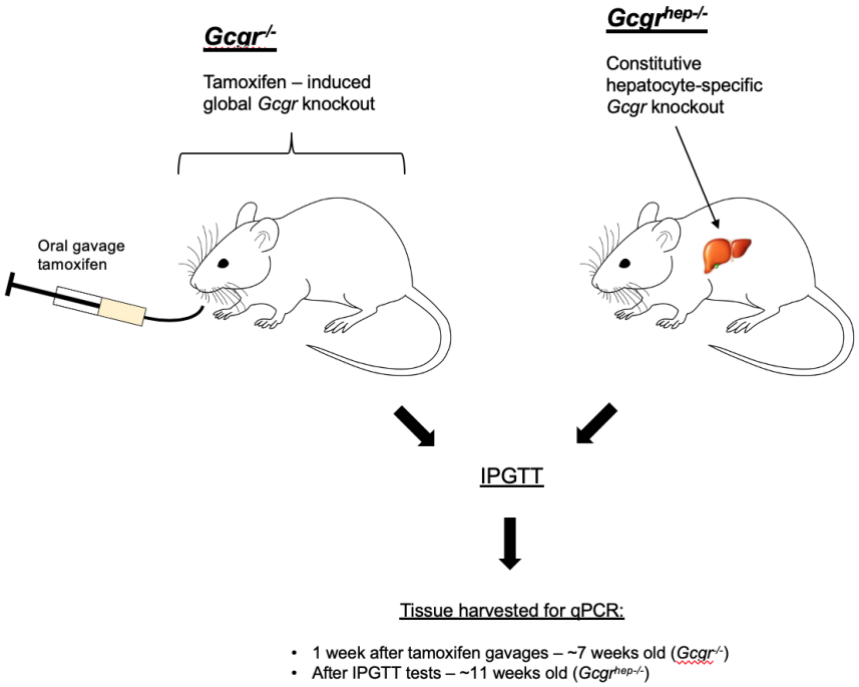

Figure 4.6 - Schematic of either constitutive hepatocyte-specific or conditional global *Gcgr*$^{-/-}$ mice

Constitutive hepatocyte-specific *Gcgr-/-* mice were generated by crossing *Gcgr*$^{fl/fl}$ mice with *Alb*-*Cre* mice. Conditional global *Gcgr*$^{-/-}$ mice were generated by crossing *Gcgr*$^{fl/fl}$ mice with Actb-Cre-ERT2 mice, and homozygous pups were administered 100mg/kg tamoxifen by oral gavage for 5 consecutive days aged six weeks and left for a week for thorough knockdown. Aged 6-weeks, *Gcgr*$^{hep-/-}$ mice were used for IPGTTs, and 4 weeks later tissue harvested for gene expression analysis. At 6-weeks, some *Gcgr*$^{-/-}$ mice were culled for tissue, whilst others were taken forward for IPGTT testing.

Whilst it was not possible to collect sufficient tissue samples for comprehensive statistical analysis, preliminary analysis (Figure 4.7) of *Gcgr* expression in different tissues indicated that *Gcgr*$^{hep-/-}$ mice showed efficient knockdown in the liver, with a small reduction in the kidney, of which the significance is unknown. Interestingly, global *Gcgr*$^{-/-}$ mice show a similar pattern of knockdown as the hepatocyte-specific knockdown Gcgr$^{hep-/-}$ mice, with less prominent changes in kidney. The small numbers used here (n=1-5) make it difficult to draw conclusions about this finding in a “global”

knockdown model but may be a consequence of differential uptake of tamoxifen after oral gavage.

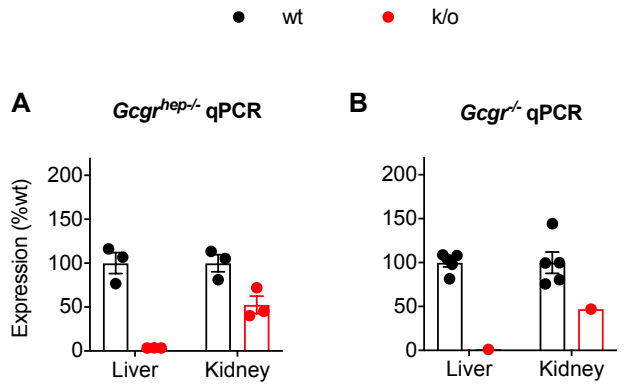

Figure 4.7 - Quantitative mRNA expression analysis of hepatocyte-specific and global *Gcgr*$^{-/-}$ mice

mRNA measured in homogenised liver or kidney samples from wild-type (wt, black) and knockout (k/o, red) mice. (A) *Alb*-Cre *Gcgr*$^{-/-}$ (n=3) and (B) *ERT*-Cre *Gcgr*$^{-/-}$ (n=1 & 5). Data presented as mean ± SEM. No statistical analysis could be performed.

IPGTTs were performed as previously described, acutely and 4 hours post peptide administration. Both male and female mice were used to allow identification of sexual dimorphism. Here, three-way ANOVA was used to analyse statistical significance between the AUCs by treatment, time (acute and 4 hour response) and genotype (wild-type or knockout). In *Gcgr*$^{hep-/-}$ wild-type males, the expected anti-hyperglycaemic advantage of SRB103Gln3 *versus* SRB103His3 at 4 hours was seemingly maintained (Figure 4.8A, top panel), although the relatively small numbers of mice used (n=5-8) meant there was no statistical difference between the AUC of the two peptides either acutely (0.46 ± 0.04 *versus* 0.48 ± 0.05M.min; three-way ANOVA, P>0.05) or after 4 hours of treatment (0.36 ± 0.04 *versus* 0.51 ± 0.09M.min; P>0.05) (Figure 4.8A, bar chart). Importantly, in keeping with the known phenotype of this mouse[685], male *Gcgr*$^{hep-/-}$ showed significantly better glucose tolerance compared to wild-type males after vehicle treatment (P<0.05) (Figure 4.8A, bar chart), making assessment of peptide treatment effects difficult to discern. Indeed, no additional anti-hyperglycaemic effect of either peptide *versus* vehicle was detectable during the acute GTT although, interestingly, the expected divergence between SRB103Gln3 and SRB103His3 after 4 hours was lost. Inter-genotype statistical comparison indicated similar glycaemia between wild-type and *Gcgr*$^{hep-/-}$ males treated with either peptide, suggesting presence or absence of the hepatic GCGR did not affect their anti-hyperglycaemic properties. Female wild-type and *Gcgr*$^{hep-/-}$ mice showed no significant difference in AUC between any treatment acutely or at 4 hours (all P>0.05), however the trends remained similar to those observed in males (Figure 4.8B).

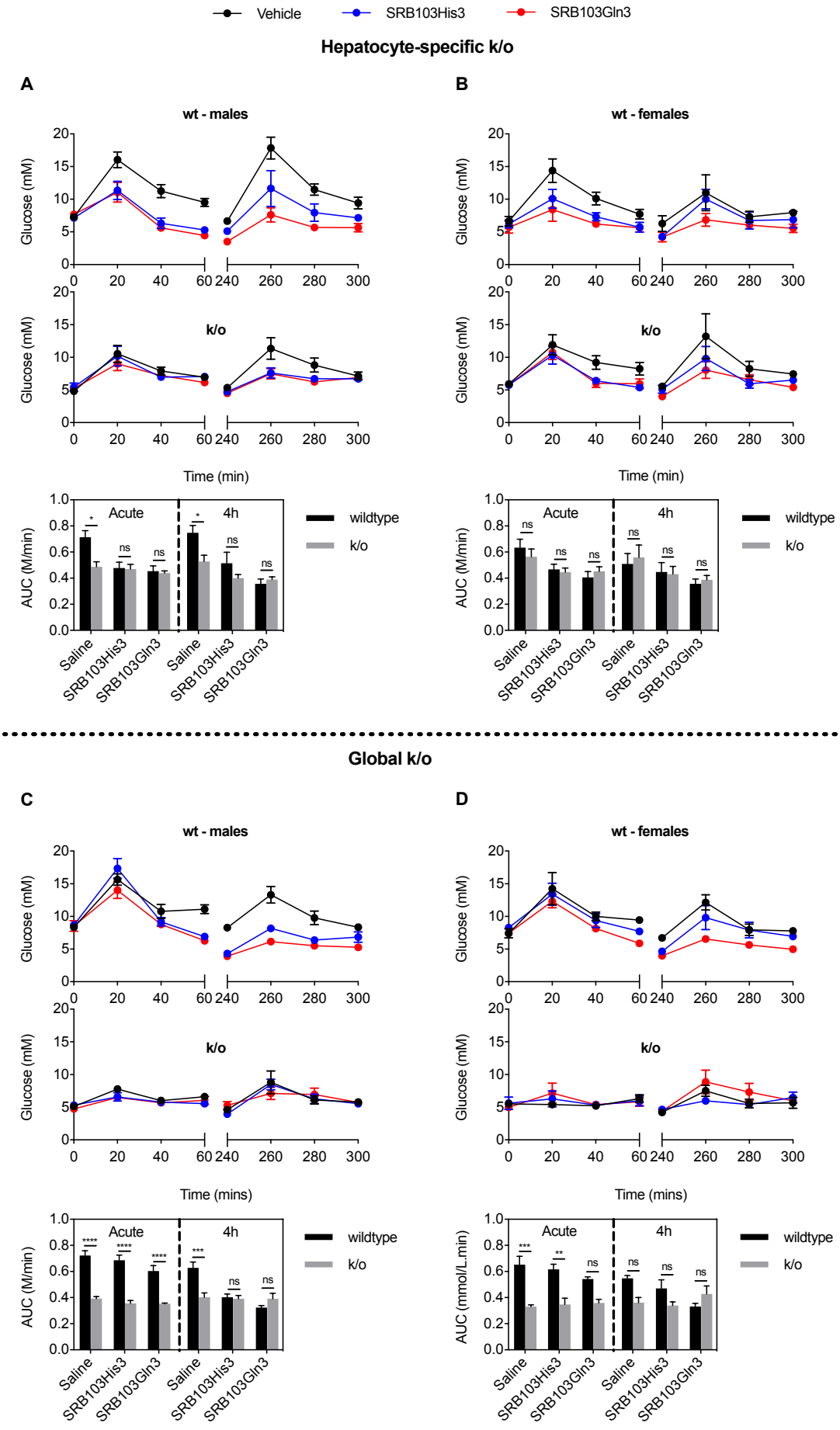
Vehicle
SRB103His3
SRB103Gln3
Hepatocyte-specific k/o
A
wt - males
B
wt - females
Glucose (mM)
k/o
Time (min)
AUC (M/min)
Acute
4h
wildtype
k/o
Saline
SRB103His3
SRB103Gln3
Global k/o
C
D
Time (mins)
AUC (mmol/L.min)

Figure 4.8 - Glucose tolerance in hepatocyte-specific or global *Gcgr*$^{-/-}$ mice

Homozygous *fl/fl* C57BL/6Jmice (6-10 weeks) were fasted for 4 hours and i.p. co-injected with 2g/kg glucose with 10nmol/kg of SRB103His3 (blue), SRB103Gln3 (red) or vehicle (black) for the acute study, and then injected with 2g/kg glucose a further 4 hours later for the 4-hour study. (A) Blood glucose levels in wild-type (top) and knockout (k/o, middle) *Alb*-Cre hepatocyte-specific *Gcgr*$^{-/-}$ male mice, with area under curve (AUC, bottom) for change in blood glucose between wild-type (black) and knockout (k/o, grey) mice, n=6/8. (B) as for (A) but in females, n=4/5 (C) as for (A) but in *ERT*-Cre whole-body *Gcgr*$^{-/-}$ males, n=5/6 (D) as for (C) but in females, n=3/4. Data presented as mean ± SEM. Significance calculated by three-way ANOVA with Tukey post hoc test. **** P<0.0001; *** P<0.001; ** P<0.01; * P<0.05; ns – not significant (P>0.05).

To investigate whether this was a liver-specific effect, *Gcgr*$^{fl/fl}$*:Actb*$^{-Cre-ERT2}$ were bred. As constitutive whole-body *Gcgr*$^{-/-}$ strains show significant islet morphological changes (e.g. alpha cell hyperplasia), as well as elevated circulating GLP-1 concentrations which can affect validity of findings[354], a tamoxifen-inducible *Gcgr*$^{-/-}$ model was used which has been shown to mitigate these adaptive but confounding factors for approximately two weeks after the loss of GCGR expression[683]. As in *Gcgr*$^{hep-/-}$ mice, global *Gcgr*$^{-/-}$ mice showed significantly reduced glycaemia after acute (three-way ANOVA, P<0.0001) and 4-hour vehicle treatment (P<0.001) (Figure 4.8C, top and middle panel). In wild-type males, acute SRB103Gln3 and SRB103His3 treatment did not have an impact on glycaemic response compared to vehicle (P>0.05), but the 4-hour treatment response showed a similar pattern to that observed previously, with SRB103Gln3 producing a greater (albeit statistically not significant due to small number used) improvement from vehicle than SRB103His3 (0.32 ± 0.02 and 0.40 ± 0.03 *versus* 0.63 ± 0.05M.min; P<0.0001 and P<0.001 respectively).

Similar to *Gcgr*$^{hep-/-}$ males, the reduction in hyperglycaemic response was markedly increased acutely for all treatments in *Gcgr*$^{-/-}$ mice, with vehicle and SRB103His3-treated mice showing the same anti-hyperglycaemic response as SRB103Gln3 treatment (P>0.05). Again, both wild-type and *Gcgr*$^{-/-}$ females showed a similar trend as seen previously, but no significant difference between the treatments at each timepoint (Figure 4.8D) (P>0.05). Differences in anti-hyperglycaemic ability between SRB103Gln3 and SRB103His3 were also not significantly different when data for both sexes was combined (P>0.05; data not shown).

In vitro data suggest a reduction in β-arrestin recruitment elicited by SRB103Gln3, at both GLP-1R and GCGR. Phenotypically, SRB103Gln3 treatment reduces glucose-induced hyperglycaemia by increasing insulin secretion (Figure 4.2C). This implies

that the recruitment of β-arrestin to either receptor in the insulin-secreting β cell might reduce the ability of dual GLP-1R/GCGR agonists to correct glucose-induced hyperglycaemia. To investigate this possibility, treatment responses in lean and DIO islet cell specific (*Pdx1*-Cre) β-arrestin-2 knockdown (*βarr2*$_{β-/-}$) mice, kindly gifted by Dr Stavroula Bitsi and Dr Alejandra Tomas (Section of Cell Biology, Imperial College London), were investigated. IPGTTs were performed at 4-hours post-treatment in lean mice, and acute and 4-hours post treatment for DIO mice.

In DIO *βarr2*$_{β-/-}$ male mice, SRB103Gln3 and SRB103His3 were similarly efficacious at producing anti-hyperglycaemic effects 4-hours post administration in both the wild-type (Figure 4.9A, top panel) and *βarr2*$_{β-/-}$ mice (Figure 4.9A, middle panel) (Three-way ANOVA; P>0.05). Contrary to the hypothesis derived here, that genetic ablation of β-arrestin-2 transcription would increase the anti-hyperglycaemic abilities of both peptides, *βarr2*$_{β-/-}$ mice appeared to show slightly (albeit without statistical significance) less of a response to either peptide compared to wild-type mice, as *βarr2*$_{β-/-}$ mice had increased but insignificant AUC compared to the wild-type mice for both SRB103His3 (0.55 ± 0.05 *versus* 0.46 ± 0.03M.min; P>0.05) and SRB103Gln3 (0.51 ± 0.06 *versus* 0.34 ± 0.03M.min; P>0.05) (Figure 4.9A, bottom panel). In females (Figure 4.9B), *βarr2*$_{β-/-}$ displayed a trend to decrease the AUC of 4h IPGTT after treatment of vehicle (0.80 ± 0.08 to 0.67 ± 0.07M.min; P>0.05), SRB103His3 (0.51 ± 0.03 to 0.39 ± 0.04M.min; P>0.05) or SRB103Gln3 (0.38 ± 0.03 to 0.34 ± 0.03M.min; P>0.05) in keeping with the role of β-arrestin-2 in controlling the response to SRB103 peptides, however none of these comparisons reached significance. In lean mice, there was a very small but not significant reduction in AUC between wild-type and *βarr2*$_{β-/-}$ in male mice treated with SRB103Gln3 (0.40 ± 0.03 *versus* 0.36 ± 0.02M.min; P>0.05) or SRB103His3 (0.58 ± 0.03 *versus* 0.56 ± 0.07M.min; P>0.05) (Figure 4.9C). This finding was corroborated in female mice (SRB103Gln3 – 0.46 ± 0.05 *versus* 0.40 ± 0.03M.min, SRB103His3 – 0.56 ± 0.04 *versus* 0.49 ± 0.03M.min; both P>0.05) (Figure 4.9D).

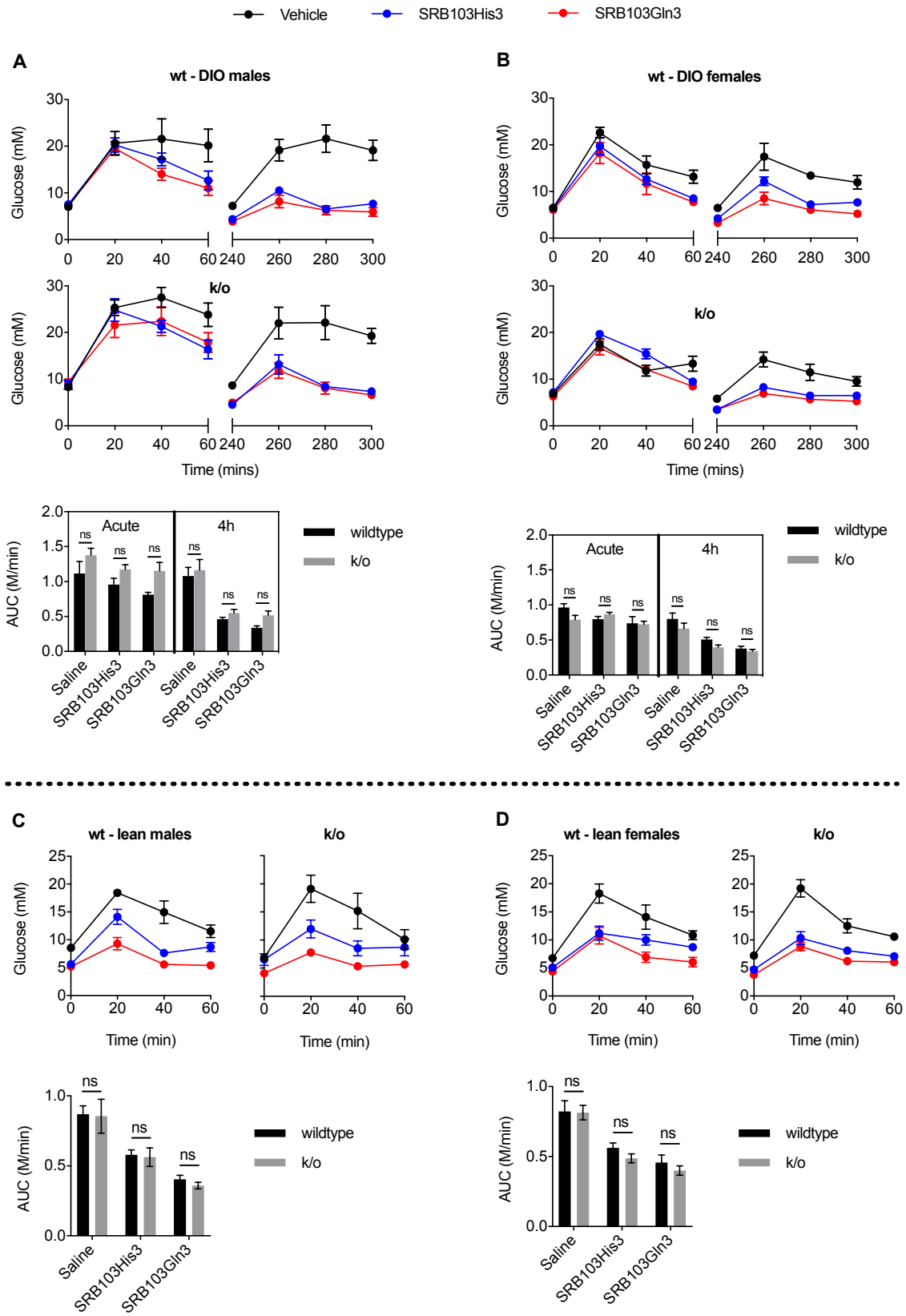
Vehicle
SRB103His3
SRB103Gln3
A
wt - DIO males
k/o
Glucose (mM)
Time (mins)
AUC (M/min)
Acute
4h
ns
wildtype
k/o
Saline
SRB103His3
SRB103Gln3
B
wt - DIO females
C
wt - lean males
Time (min)
D
wt - lean females

Figure 4.9 - Glucose tolerance in lean and DIO *βarr2*$_{β-/-}$ mice

βarr2$_{β-/-}$ C57BL/6J mice were fasted for 4 hours and i.p. co-injected with 2g/kg glucose with 10nmol/kg of SRB103His3 (blue), SRB103Gln3 (red) or vehicle (black) for the acute study, and then injected with 2g/kg glucose a further 4 hours later for the 4-hour study. (A) Blood glucose levels in wild-type (top) and knockout (k/o, middle) DIO *Pdx1*-Cre β cell-specific *βArr*$_{-/-}$ male mice (7-9 months), with area under curve (AUC, bottom) for change in blood glucose between wild-type (black) and knockout (k/o, grey) mice, n=5/6. (B) as for (A) but in females, n=5/7. (C) as for (A) but in lean mice (4-6 months) and only a 4-hour study was performed, n=5/7. (D) as for (C) but in female mice, n=9. Data presented as mean ± SEM. Statistical analysis performed as either three-way ANOVA [(A) & (B)] or two-way ANOVA [(C) & (D)] with Tukey post hoc test. Ns- not significant (P>0.05).

Collectively, these data do not identify a critical role for β cell β-arrestin-2 in the control of whole body glucose[736]. Moreover, no significant or reproducible improvement in anti-hyperglycaemia was observed with SRB103 peptide treatment in *βarr2*$_{β-/-}$ knockdown mice, suggesting β-arrestin mediated effects are not a significant factor in the prolonged anti-hyperglycaemic phenotype witnessed of SRB103Gln3.

4.2.5 Acute anorectic effect of SRB103 peptides in lean and DIO wild-type mice

Studies presented above illustrate how SRB103Gln3 demonstrates promising anti-hyperglycaemic properties, suggesting biased dual agonists can indeed improve therapeutic efficacy. To determine whether SRB103Gln3 could induce changes in food intake as it did with anti-hyperglycaemia, lean and obese C57BL/6J wild-type mice were fasted overnight and energy intake after refeeding was measured the next day, 30 minutes after peptide administration.

Lean mice treated with SRB103His3 and SRB103Gln3 showed no statistically significant difference in food intake by the end of the 8-hour study (Two-way ANOVA, P>0.05) (Figure 4.10A), but there was a clear trend favouring enhanced anorectic efficacy for SRB103Gln3 treatment. In lean mice, SRB103Gln3 was the only treatment which resulted in a significant reduction in 8-hour food intake compared to vehicle treated mice (P<0.01). Four more pairs of Gln3/His3 pairs were assessed for their ability to control acute food intake in lean mice (Table 4.2). However, only one of the Gln3 dual agonists causing a significant reduction in food intake compared to the His3 corresponding peptide (P<0.05).

In obese mice, SRB103His3 and SRB103Gln3 treatment significantly reduces food intake compared to vehicle over the 8-hour period recorded (P<0.001 for SRB103His3; P<0.0001 for SRB103Gln3). Moreover, similar to observations in lean mice, SRB103Gln3 treated mice showed a greater reduction in food intake compared to SRB103His3 treated mice but did not reach significance (0.6 ± 0.1g *versus* 1.0 ± 0.2g respectively; P>0.05) (Figure 4.10B).

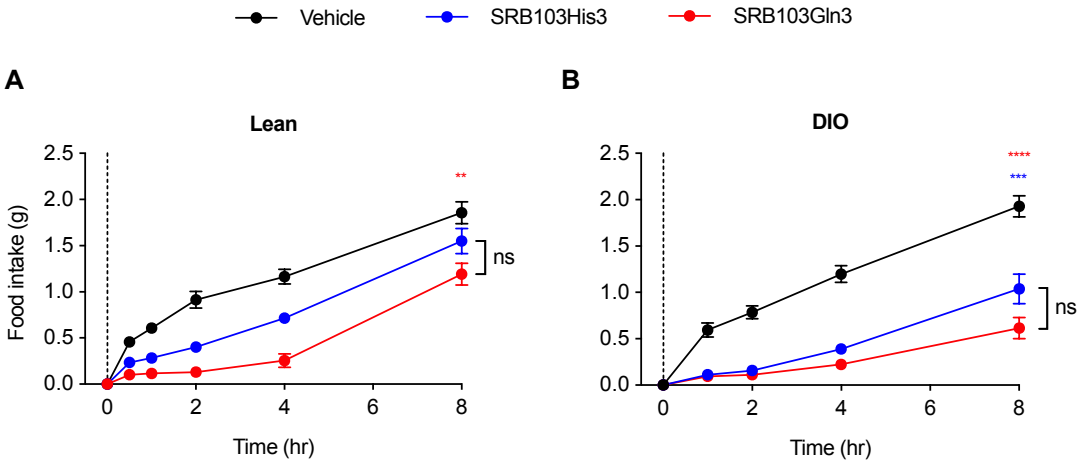

Figure 4.10 - Effect of SRB103 peptides on acute food intake in lean and DIO mice

Male C57BL/6 mice were fasted overnight and injected i.p. with 10nmol/kg SRB103His3 (blue), SRB103Gln3 (red) or vehicle (black) 30 minutes before refeeding (dotted line). (A) food intake in lean mice (3 months, n=8). (B) as for (A) but in DIO mice (6 months, n=10). Food intake corrected from baseline food weight. Data presented as mean ± SEM. Statistical analysis performed by two-way ANOVA with Tukey post hoc test. **** P<0.0001; *** P<0.001; ** P<0.01; ns – not significant (P>0.05).

	His1	Gln1		His2	Gln2		His3	Gln3		His4	Gln4	
Food (g)	2.6 ± 0.1	1.9 ± 0.1	*	1.9 ± 0.1	1.6 ± 0.1	ns	1.9 ± 0.2	1.9 ± 0.1	ns	2.0 ± 0.1	1.9 ± 0.1	ns

Table 4.2 - Effect of four more Gln3/His3 dual GLP-1R/GCGR agonists pairs on food intake in lean mice. Mice were fasted overnight and refed standard chow 30 minutes post i.p. administration of 10nmol/kg His3/Gln3. Food intake was recorded over a period of 8 hours, n=10. Data presented as mean ± SEM. Data analysed by two-way ANOVA with Tukey post hoc test. * P<0.05; ns – not significant (P>0.05).

This suggests that $G\alpha_s$-favoured signalling of dual GLP-1R/GCGR agonists affects the control of food intake and should be considered when analysing their effect on general energy metabolism for the treatment of obesity and associated metabolic diseases.

4.3 Discussion

In this chapter, the two dual agonists identified in the previous chapter, SRB103Gln3 and SRB103His3, were examined *in vivo* to investigate the effect of cAMP/$G\alpha_s$-favoured signalling with dual GLP-1R/GCGR agonists in the context of energy homeostasis. Identification of receptor dependency for observed physiological effects was then attempted using both pharmacological and genetic approaches. The main results from this chapter are as follows:

1) SRB103Gln3 and SRB103His3 are equally efficacious at reducing hyperglycaemia acutely in lean and DIO mice.
2) After prolonged treatment (4 or 8 hours) SRB103Gln3 enhanced anti-hyperglycaemic efficacy compared to SRB103His3.
3) Molecules designed to antagonise the receptor, or dual agonists designed to specifically activate one receptor and not the other, were not efficient at elucidating which receptor was responsible for the anti-hyperglycaemic effects.
4) In hepatocyte-specific and global *Gcgr*$^{-/-}$ mice, vehicle and SRB103His3 treatment results in improved anti-hyperglycaemic response. *Barr*$_{\beta}$$^{-/-}$ mice displayed no difference in anti-hyperglycaemic control.
5) There is a small but not significant reduction in energy intake after SRB103Gln3 administration compared to SRB103His3.

4.3.1 SRB103Gln3 is more anti-hyperglycaemic than SRB103His3 at extended timepoints

Previously, it was shown that Ex-Phe1, a weak GLP-1RA with minimal β-arrestin recruitment, possesses improved anti-hyperglycaemic efficacy after longer treatment times[276], correlating with Ex-Phe1 increased insulin secretion at these later timepoints due to reduced GLP-1R desensitisation and/or internalisation. In the previous chapter, SRB103Gln3 showed reduced β-arrestin recruitment at both the GCGR and GLP-1R (Figure 3.4); therefore, the possibility is raised that SRB103Gln3 may support more prolonged signalling and therefore more prolonged metabolic effects via action at either receptor. In mice, after acute peptide treatment, anti-hyperglycaemic efficacy of SRB103His3 and SRB103Gln3 were similar during an IPGTT (Figure 4.2A) but, after

4 hours and 8 hours of peptide treatment, SRB103Gln3 showed superior anti-hyperglycaemic abilities. This is in keeping with findings from the previous chapter, in which it was postulated that prolonged cAMP signalling in Huh7 cells after SRB103Gln3 treatment arose from reduced GCGR desensitisation and/or internalisation. The fact that SRB103His3, which maximally recruits β-arrestins at both receptors, performs as poorly as vehicle 8 hours post injection (whereas SRB103Gln3 is still therapeutically active) might be attributable to desensitisation. As there is no difference in PK, it might be suggested that SRB103His3 causes receptor desensitisation/internalisation to the point that circulating agonist concentrations fall below a threshold required to engender an anti-hyperglycaemic response.

The mechanism by which SRB103Gln3 exerts its superior anti-hyperglycaemic effects appears to be via increased insulin secretion, as suggested in Figure 4.2C. Here, SRB103Gln3 increased insulin secretion four-fold 4 hours after peptide administration compared to SRB103His3. Neither peptide caused a significant rise 8 hours post peptide treatment. This is surprising given the fact SRB103Gln3 still elicits anti-hyperglycaemic effects but may be explained by increased insulin sensitivity caused by a total of 9 hours of fasting (1-hour pre peptide and 8 hours during peptide administration), or by poor precision of the HTRF at minute concentrations masking small iterations in insulin secretion. Other long acting incretin therapies have observed much greater dose-dependent insulin secretion in mice at time points extending beyond 8 hours[455,737], suggesting the SRB103 peptides are almost fully degraded in the system by 8 hours but enough remains for SRB103Gln3 to be anti-hyperglycaemic.

Due to the recent finding that GCGR activation can lead to a paradoxical increase in insulin sensitivity[394], ITTs were performed after administration of SRB103 peptides to identify if prolongation of GCGR signalling by SRB103Gln3 might underpin its more sustained anti-hyperglycaemic effect. However, no difference in insulin sensitivity was observed in lean mice at three separate doses of insulin (Figure 4.2E).Given the peptide-specific differences in blood glucose before the insulin bolus was administered (i.e. 4 hours post agonist administration), a more sophisticated technique such as hyperinsulinaemic clamping may be required to confirm whether subtle differences in insulin sensitivity are evident after treatment with either SRB103 compound. Whilst GCGR activation is linked to increased insulin sensitivity[394], G protein-bias at the GLP-

1R has been shown to not affect ITT-mediated insulin tolerance[502]. This would agree with the results from the NanoBit assay which suggests SRB103Gln3 more actively signals toward $G\alpha_s$ compared to SRB103His3 at the GLP-1R, and neither SRB103 peptide shows any difference in insulin sensitivity in their ITT.

Differences in pharmacokinetics could potentially confound the difference in 4- and 8-hour anti-hyperglycaemic effect between SRB103Gln3 and SRB103His3. DPP-IV acts at the second amino acid in OXM[730,731], on which the SRB103 peptide sequences are based, although the AIB substitution in the SRB103 peptides is expected to confer significant protection against DPP-IV proteolysis[738]. Nevertheless, it was important to investigate whether altering the third amino acid (Gln3 *versus* His3) affected DPP-IV activity. However, 4-hour plasma samples showed similar circulating levels of both peptides and both SRB103 peptides show similar DPP-IV lysis profiles in vitro (Figures 4.2F & G). Whilst this method of DPP-IV efficacy analysis isn't completely valid, as it does not encompass first-pass metabolism into account as well as other physiological factors, it represents a basic model of peptide breakdown which is useful to show large differences in intrinsic DPP-IV effects.

In the present series of studies, no GLP-1 mono-agonist was used in the acute studies as a comparator. It would have been interesting to investigate how SRB103Gln3 compared to GLP-1R agonists marketed for diabetes treatment, such as exenatide, semaglutide or liraglutide, or even a biased GLP-1R agonist such as Ex-Phe1. Acutely, dual agonists have been shown to display similar anti-hyperglycaemic abilities to GLP-1R agonists[227,228], but this has not been investigated using a delayed IPGTT approach to allow for differences in receptor desensitisation. Similarly, a lean cohort was not used as a secondary control (alongside vehicle as a primary control) for the DIO studies. It would be interesting to repeat the acute IPGTTs with lean and DIO comparators to discern the effect of high fat feeding on glycaemia, and explain whether the similar baseline glycaemic levels observed between lean and DIO mice was a true observation, or due to stress exacerbating the lean mice baseline glycaemia (as is more likely).

4.3.2 Pharmacological approaches fail to confirm which receptor is responsible for the SRB103Gln3 phenotype

The results discussed in section 4.3.1 highlight distinct insulinotropic capabilities of the SRB103 peptides arising at prolonged treatment periods. The insulinotropic and anti-hyperglycaemic properties of SRB103Gln3 resemble those of the G protein-biased GLP-1R agonist Ex-Phe1[276]. However, NanoBiT recruitment assays suggest that SRB103Gln3 shows selective reductions in β-arrestin-2 recruitment at the GLP-1R and partial agonist at the GCGR (Figure 3.4C & D), whereas PathHunter and cAMP assays suggest SRB103Gln3 is biased at the GCGR and a balanced agonist at the GLP-1R (Figure 3.3A). Whilst GLP-1R is classically associated with augmenting β cell insulin secretion, GCGR agonism has also been linked to increased insulin secretion both *in vivo* and *ex vivo*[12,390,391,394], raising the possibility that the phenotype described in section 4.2.1 could originate from effects at either or both receptors.

In this study, long-lasting antagonists (Acyl-DHG and Acyl-Ex9) were designed based on previously used DesHis1-[Glu9]-glucagon(1-29) amide[733] and exendin9-39[732] to inhibit the individual receptors to confirm receptor contributions to the phenotype. The extended fatty acid was attached to the C-terminus of the peptide as to not affect binding of the peptide to the orthosteric site. However, *in vitro* analysis showed the antagonist activity of both ligands was mild at best when cognate ligand (Figures 4.4A & B). In fact, acyl-DHG acted as a weak GCGR agonist, therefore there was no observable concentration of Acyl-DHG which both inhibited glucagon and did not itself activate the GCGR. Therefore L-168,049, an oral, non-competitive antagonist, was investigated as an alternative. Despite displaying promising *in vitro* antagonism of glucagon (Figure 4.4C, left), and a high reported affinity for the GCGR, L-168,049 surprisingly increased hyperglycaemia compared to glucagon alone in mice (Figure 4.4C, right). It has been reported that L-168,049 is approximately twenty-fold weaker at the murine GCGR compared to human GCGR with limited abilities to block glucagon-induced hyperglycaemia, even at 50mg/kg[734]. As the CHO cells used for *in* vitro verification express the human receptor and mice express a different isoform of the receptor, this disparity in antagonist affinity was missed. This emphasises the necessity to validate *in vitro* and *in vivo* data in the same species' receptor.

The secondary approach was therefore trialled based on receptor-selective peptides was opposing to the idea of blocking signalling at one receptor to visualise the effects of the SRB103 peptides at the other receptor. Here, the rationale was to produce peptides based on SRB103 which contained the Gln3/His3 switch but favoured signalling at one receptor over another. In the cAMP screening assays using analogous peptides, addition of tyrosine at position 1 and serine at position 18 resulted in ablation in signal at the GLP-1R and GCGR respectively. Ideally, two dual agonists with tyrosine at position 1 and either Gln3 or His3 would result in two agonists which maintained the GCGR phenotype previously seen but equally lacking potency at the GLP-1R. However, Gln3Tyr1 displayed twenty times greater potency at the GLP-1R compared to His3Tyr1, which would be a greatly confounding factor in *in vivo* glycaemic control between the two Tyr1 peptides. Whilst unpredictable, this failure is not surprising. As discussed in the previous chapter, single amino acid substitutions along the ligand will affect its tertiary structure and consequently the quaternary ligand-receptor complex, and in the case of dual agonists, this can be specific to one receptor over another.

Reversible antagonists have benefits over transgenic lines as they don't have underlying physiological differences, therefore antagonists are suitable to look to repeat with more time available. Different methods of blocking the GCGR are available, which could be investigated in the future. Monoclonal antibodies have been used which show robust antagonistic abilities at the murine GCGR[739,740], and other non-competitive GCGR antagonists are currently in development[734]. At the GLP-1R, non-competitive antagonists are available with extended pharmacokinetics, and is validated as blocking Ex4-mediated insulin secretion in rats[741].

4.3.3 Hepatocyte-specific knockdown of Gcgr increases glucose tolerance

Despite previous studies highlighting the efficacy of commercially available antagonists and the attempted development of receptor selective dual agonist switch maintained the biased signalling profile seen previously, it was still not possible to elucidate which receptor was responsible for the phenotypic differences observed. Therefore, hepatocyte-specific or conditional global knockdown mice were generated

to identify the GCGR-dependant component of therapeutic response to SRB103 peptides (Figure 4.7). GCGR knockdown mice have been widely used to validate GCGR antagonists as potential anti-diabetic therapies, to investigate glucagon physiology[283,388,682,685] and to delineate the role of the glucagon receptor with dual GLP-1R/GCGR agonist treatment[437,729].

In both *Gcgr*$^{hep-/-}$ and *Gcgr*$^{-/-}$ mice a major improvement in overall glycaemic control was observed (Figure 4.8 and 4.9). Previously, it has been shown that both global knockdown and hepatocyte-specific GCGR knockdown mice display compensatory mechanisms which produce a physiological change such as β cell hyperplasia and increased circulating concentrations of glucagon and GLP-1[354]. These will likely all contribute to the glycaemic phenotype seen in both *Gcgr* knockdown lines used in the present study. As seen in lean and DIO mice previously, SRB103Gln3 treatment produces a repeated (but not statistically significant) improvement in anti-hyperglycaemic effects than SRB103His3 after 4-hours of treatment in wild-type males and females (Figure 4.8). However, any divergence between SRB103His3 and SRB103Gln3 at 4-hours was lost in all knockout groups (hepatic/global and male/female), suggesting that in the wild-type mice, signalling at the GCGR (or lack thereof if SRB103Gln3 is a partial agonist) is important to the therapeutic phenotype of SRB103Gln3. Again, it is key to reiterated that GCGR knockout mice display significantly improved glucose tolerance, therefore it is harder to detect any agonist-related differences between SRB103His3 and SRB103Gln3. Therefore, the lack of any difference in observed response is not necessarily indicative of the fact there is no effect (i.e. it could be a false negative).

Both *Gcgr* knockdown models used here displayed lower glycaemia than their wild-type littermate controls. In both males and female *Gcgr*$^{hep-/-}$ mice, SRB103 peptide treatment resulted in greater (but not significant) anti-hyperglycaemic effects 4-hours post-peptide administration compared to vehicle treatment, when the divergence in peptide effect is most apparent (Figure 4.8A & B, middle panel). Opposingly, in the *Gcgr*$^{-/-}$ mice, no treatment difference was observed between peptide- or vehicle-treated mice at the 4-hour timepoint, as the glucose bolus produced a smaller peak response in the vehicle-treated group compared to *Gcgr*$^{hep-/-}$. This suggests that the improved glycaemia in the *Gcgr*$^{-/-}$ could relate to ablated GCGR signalling outside of

the primary hepatic site of GCGR activity. However, as discussed previously, the other alternative for this finding is that there is an increased secretion of GLP-1, which has been shown to be upregulated after *Gcgr* knockdown[354], resulting in greater incretin-mediated improvements in glucose control. However, as SRB103Gln3 displayed increased insulinotropic abilities, a β cell-specific *Gcgr* knockdown strain should also be used to uncover whether the mechanism by which SRB103Gln3 exerts a greater insulinotropic effect is mediated by signalling at the GCGR, or GLP-1R. Studying the contribution of GCGR signalling at the β cell in the context of dual agonists has not been performed before, therefore expanding this to implicating bias as well would be exciting.

The current study also replicated the finding that female wild-type mice display a slight improvement in glucose control compared to males[742-744]. Here, using AUC as a measurement of total glucose tolerance, vehicle-treated wild-type female mice display repeated, but non-significant, improvements in glucose control *versus* their male littermate equivalents (P>0.05). In the $Gcgr^{hep-/-}$ cohort, a mean AUC during IPGTT of 0.71 ± 0.05M.min *versus* 0.63 ± 0.13M.min was observed for wild-type males and females respectively after acute vehicle administration; the equivalent IPGTT AUC results after an additional 4-hours fasting was 0.75 ± 0.06M.min *versus* 0.51 ± 0.16. A similar comparison in the $Gcgr^{-/-}$ cohort, where wild-type mice show AUCs of 0.72 ± 0.04M.min *versus* 0.65 ± 0.06M.min acutely and 0.63 ± 0.05 *versus* 0.55 ± 0.2M.min after 4-hours for wild-type males and females. In the present study, female mice were included to confirm sex dimorphism in the context of dual GLP-1R/GCGR agonists, which hasn't been reported before. Additionally, inclusion of females was used to increase statistical significance, as male mouse numbers were lower than that performed in earlier studies in wild-type mice. However, addition of female mice failed to improve the resolution of statistical significance (data not shown). Mechanisms behind the apparent dimorphism were not investigated, but there is evidence for incretin and neuroendocrine response dimorphism both mice and humans which affect glucose homeostasis. Firstly, human studies show that, for an identical glucose dose, women display greater levels of insulin secretion and sensitivity[745]. In mice, Yassine *et al.* showed a common human GIPR single nucleotide polymorphism (Q354), results in greater systemic glucose control in females but not males (paper not published yet). Humans display sexual dimorphism is present with regards to incretin-mediated

insulinotropic effects[746], where females display greater diabetic correction and weight loss after year-long exenatide treatment[747]. Finally, disruption of GLP-1-mediated control of glucagon secretion appears to more greatly affect female mice than males, suggesting females display greater suppression of postprandial glucagon secretion through a GLP-1-mediated pathway, whereas males may be able to compensate through other pathways[748]. Cumulatively, these studies highlight separate mechanisms by which females used in the present study may be

As SRB103Gln3 is predicted to elicit its effects at extended time-courses due to reduced β-arrestin-mediated target receptor desensitisation and/or internalisation in the β cell-specific, β-arrestin knockdown mice were also assessed. If SRB103Gln3 is more anti-hyperglycaemic after chronic treatment due to reduced β-arrestin-mediated internalisation, SRB103His3 should be more anti-hyperglycaemic when β-arrestin is knocked out. Pancreatic β-arrestin-1 and -2 mice (using *Pdx1*-Cre) have been shown to have greatly impaired insulin secretion compared to wild-type mice[281,736]. Interestingly, in a previous study in which β-arrestin-2 was specifically knocked out in β cells, differences in glucose tolerance was only observed in male DIO mice and not lean[736]. In the present study, however, no genotype-derived difference in baseline glucose tolerance was seen in either lean or DIO mice. Other pancreatic-specific Cre drivers have been reported and these depend on species, cell-type and developmental status of the progenitor cell, however there can be “leaky” expression of these primers in central tissue[749]. *Pdx1* is expressed in the pre-pancreatic endodermal cells and is therefore expressed in all pancreatic tissue, however there is also minimal recombination in duodenum, stomach and hypothalamus[750,751]. Regardless, it provides stringent pancreatic-wide knockdown of the gene. Initial expression analysis provided by Dr Stavroula Bitsi show that approximately 60% knockdown is achieved in the whole islet, which likely translates to near total knockdown in the β cell population once the contribution from other islet cell types is factored in. A compensatory increase in pancreatic β-arrestin-1 may account for a lack of effect in β-arrestin-2 knockdown mice, however this has not been reported in the *Pdx1-Cre* β-arrestin-2 knockdown mouse[736]. Nevertheless, producing a double β-arrestin knockdown line may be necessary.

There are many future experiments that the transgenic mice described here would be useful for. With regards to IPGTT studies, increasing the numbers of mice used in the acute IPGTTs would be useful to further prise any statistical significance between wild-type and knockdown mice. It would also be valuable to test the SRB103 peptides in GLP-1R mice to confirm if the acute effects of SRB103Gln3 treatment are linked to GLP-1R signalling. Dual GLP-1R/GCGR agonists have been shown to become more hyperglycaemic when the GLP-1R is knocked out[631], and thus it has been interpreted that all anti-hyperglycaemic abilities of dual agonists are chiefly related to its pharmacology at the GLP-1R. There is a gap in literature in looking at the effect of acute glucose tolerance in mice where either the GCGR or GLP-1R has been knocked out, especially in the context of biased dual agonists.

4.3.4 SRB103Gln3 tends to increase satiety in lean and obese mice

Another therapeutic parameter that is important to consider for anti-obesity/anti-diabetic drugs, such as dual GLP-1R/GCGR agonists, is their effect on energy intake. From a therapeutic perspective, anorectic effects observed in acute feeding studies can be interpreted in opposing ways. Firstly, reduced food intake can be seen as positive as it reduces net caloric intake which leads to weight loss, itself linked to improvements in diabetes[129-132]. alternatively, marked reductions in acute food intake might be indicative of nausea, the main adverse effect of GLP-1R agonists[326]. Moreover, feeding is associated with activation of hedonistic and reward pathways[752], and most diets fail when the central caveat of the diet is reducing portion size[753]. A drug that makes the patient feel unwell and lose the pleasurable aspects of eating could lead to poor compliance.

In this chapter, acute food intake between the low efficacy compound SRB103Gln3 and balanced SRB103His3 was measured in both lean and DIO mice (Figure 4.10). Interestingly, there was no statistical difference in food intake between SRB103Gln3 and SRB103His3 in either model. There was, however, an observable trend of reduced food intake in SRB103Gln3-treated mice, and in lean mice at 4 hours post injection there was a significant difference in food intake between mice injected with the two peptides ($P<0.001$). This may suggest that the effect of the dual agonists is

depleting after 4 hours as it gets degraded, and therefore food intake in SRB103Gln3-treated mice compensates by catching up to SRB103His3-treated mice.

A major caveat with this study is that it was performed in the light phase, when mice are typically sedentary[754]. Indeed, resting (light) phase testing has been shown also to negatively impact behavioural and social parameters in mice, resulting in reduced food intake relative to dark phase testing[754]. This may be bypassed by allowing adaptation to the shift in feeding[755]. In this study, resting phase testing may mask the true effect of SRB103 peptides on food intake behaviour. It would be interesting in future to investigate SRB103 effects on satiety in the dark phase to confirm no difference in normal feeding behaviour between the biased and unbiased compound.

The mechanism as to why SRB103Gln3 treatment tends to reduce food intake is likely GLP-1R mediated. It is disputed as to whether glucagon or GCGR agonists reduce food intake or not. Effects of GCGR agonism on food intake are likely dose-related (i.e. high dose leads to nausea which leads to reduced food intake) as opposed to GCGR having any true physiological link to food intake. GLP-1R activation causes satiation, which results in reduced food intake. However, three examples of cAMP- or Gαs-biased GLP-1R agonists do not significantly reduce food intake compared to balanced agonists[276,502,520]. There are two different possibilities for this divergence in effect of biased GLP-1R signalling. Firstly, it may be that differences are related to tissue bias between the β cell, the site of insulin secretion, and neurons, where food intake is controlled. If $G\alpha_s$ or cAMP bias is present at the β cell but not neuronally, then SRB103Gln3 would not be expected to produce a difference in food intake compared to SRB103His3. The second, less likely theory is that SRB103His3 can more readily cross the blood-brain barrier (BBB) than SRB103Gln3, and therefore the lack in sustained signalling evident in SRB103His3 is offset by its ability to more readily stimulate central GLP-1R. This would make sense as the BBB consists of epithelial cells which separate blood from cerebrospinal fluid (CSF)[756]. If SRB103His3 can more readily recruit β-arrestin, and thus internalise more rapidly, it may more readily pass through this endothelial cell layer. However, work performed in the laboratory has suggested that transport across the BBB is not concomitant with β-arrestin-mediated internalisation. This could be tested in the future by either collecting CSF and performing an RIA to confirm circulating concentrations in the central nervous system

(CNS) or performing in vitro characterisation of the SRB103 peptides' abilities to pass through the BBB.

Whilst not central to the main research themes at hand, repeating these acute feeding studies in the GLP-1R or GCGR knockdown mice could be performed in future to confirm the receptor responsible for the phenotype observed.

4.3.5 Summary

To summarise this chapter, it has been shown that signalling bias with dual GLP-1R/GCGR agonists results in an improvement in prolonged anti-hyperglycaemic response. This translates into a disease model and is therefore a relevant and interesting finding. The mechanism by which this likely occurs is through a diminished glucagon receptor signalling and potentially also bias at the GLP-1R (however, this is still to be confirmed). There are no significant effects seen with satiety and therefore the effect of the biased dual agonist is possibly pancreatic, however diminished hepatic signalling is another possibility. Moving forward, the next steps will be to translate the beneficial acute *in vivo* effects of SRB103Gln3 on glucose tolerance and food intake into a chronic study.

5 Chronic effects of biased GLP-1R/GCGR dual agonist SRB103Gln3

5.1 Introduction

5.1.1 Improving incretin therapy pharmacokinetics

Native GLP-1 and OXM have circulating half-lives in humans of approximately 2 and 12 minutes in humans respectively[237,423,424], making these hormones impractical as a therapeutic due to their rapid degradation. Therefore, two things are essential for chronic administration of an incretin drug. Firstly, it must obviously remain efficacious throughout the duration of dosing to both cause and maintain weight loss and reduce hyperglycaemia. Secondly, it is critical that the drug has a long residing half-life to reduce the number of administrations required, increasing the patients' quality of life and the probability of patient compliance. The discovery of Ex4 in 1992 from the venom of *Heloderma suspectum* accelerated the therapeutic potential GLP-1R mono-agonists.

Ex4 has a circulating half-life of between 26 minutes and 2 hours in humans[299,757]. Ex4, a 39 amino acid peptide, has 53% homology to human GLP-1[296], demonstrating that altering the amino acid sequence of GLP-1 can result in a peptide with reduced susceptibility to peptidase degradation. This increase in half-life was achieved with no loss of potency, as Ex4 has similar receptor potency to GLP-1 at the GLP-1R[758]. In humans, Ex4 administration results in a marked and sustained improvement in glucose tolerance in both non-diabetic and diabetic patients[299,301,759,760]. Therefore, a twice-daily injectable form, exenatide (Byetta®), was approved as an adjuvant for metformin or sulphonylurea treatment in diabetic patients in 2005[301,760]. Another example of a GLP-1R agonist with alterations in amino acid sequence to improve pharmacokinetics and pharmacodynamics is lixisenatide, a 44 amino acid peptide with the first 38 amino acids of Ex4 and a 6 lysine residue tail[761]. Like exenatide, the amino acid additions slightly increase the circulating half-life to approximately 3 hours[762]. Lixisenatide also shows four times greater potency to the GLP-1R compared to GLP-1[761,763]. Clinical trials show that, in diabetic and non-diabetic humans, daily injections of lixisenatide for 12 to 52 weeks results in a vast improvement in insulin-secretory response, HbA1c measurements and glucose regulation[764-767]. Interestingly, in a study comparing lixisenatide (once-daily) to exenatide (twice daily), lixisenatide reduced

postprandial glucose levels to a greater extent and was considered more tolerable, with fewer side effects[768].

The more similar a drug is to the endogenous ligand, the less likely it is to elicit an immunogenic response, which is a clear benefit of designing a peptide-based drug centred around amino acid substitutions. Compared to other methods used to extend circulating times, the relative size of amino acid switches is minute compared to non-peptidergic approaches taken. This reduces steric hindering of ligand-receptor binding and hence produces a more potent peptide[769,770]. However, amino acid substitutions are still liable to peptidase degradation, and the free circulating drug is still metabolised rapidly through renal and hepatic clearance mechanisms[771]. Therefore, the extent to which pharmacokinetics can be extended just by amino acid switches is limited, when compared to the non-peptidergic alterations discussed below.

The drugs noted above require at least once-daily administration, if not twice daily. Due to the short, wave-like PK profiles of these once-daily drugs and the repeated administration required to maintain therapeutic doses of exenatide and lixisenatide, patient compliance can be reduced. Therefore, new strategies outside of amino acid substitutions are used in this drug class to extend PK to reduce administration frequency. Liraglutide became FDA approved as a treatment for type 2 diabetes in 2010, and for obesity in 2014. It shares 97% sequence homology to native GLP-1, different only in a lysine is substituted for arginine at position 34 and palmitic acid is attached by a glutamoyl spacer on the lysine at position 26[772]. Interestingly, addition of the fatty acid does not impede potency at the human GLP-1R, something which limits many sidechain additions[772]. The resulting peptide is 98% bound to albumin and has an elimination half-life of 13 hours in human, however it is still only suitable for once-daily dosing[773]. Semaglutide is considered the next-generation of GLP-1R agonists and is possibly the most exciting current GLP-1R agonist on the market. With an amino acid structure similar to liraglutide, semaglutide also contains AIB at position 2 and a dicarboxylic C18 fatty acid attached to the lysine. The addition of the dicarboxylic fatty acid is innovative for this drug class, allowing for increased binding affinity to albumin, and increases elimination half-life to 7 days[774]. This makes semaglutide a once weekly s.c. injectable GLP-1R agonist[775], and the steady circulating state makes it less likely to induce acute nausea which may improve patient

compliance. Semaglutide has also been approved as an oral drug for T2DM treatment, however due to extensive GI degradation, it is administered at approximately 100 times the dose as the injectable form and is taken once daily. Two further GLP-1R agonists of note are albiglutide and dulaglutide. The former consists of two GLP-1(7-37) molecules linked by human albumin, resulting in a 645 amino acid product, while the latter is a GLP-1(7-37) molecule conjugated to an IgG4 immunoglobulin[776,777]. These peptides produce good elimination half-lives of five to seven days[778,779]. Additional peptide modifications such as acylation[273], addition of cholesterol moieties[437] and PEGylation[436] have similarly been utilised with dual GLP-1R/GCGR agonists. Whilst the addition of large molecules to small peptides is possible, and such peptides exhibit clinical evidence for weight loss and diabetes control, large extra-peptidergic molecules often compromise receptor affinity and potency for improved circulating half-life. Therefore, further investigation is required to produce a long-circulating, potent peptide agonist.

The final GLP-1R agonist to discuss is taspoglutide, which was the first once-weekly GLP-1R agonist to be evaluated in clinical trials[780]. The chemical structure of taspoglutide is simply substitution of AIB into GLP-1(7-36) at positions 8 and 35, sites of protease degradation[781]. However, what makes taspoglutide novel is the integration of a zinc chloride ($ZnCl_2$) diluent, creating a s.c. depot allowing for slow release of the peptide into the circulation[780]. In humans phase II trials, taspoglutide displayed significant improvements in anti-hyperglycaemic and weight loss abilities with metformin[780,782] and improved HbA1c profiles compared to exenatide[783]. However, phase III investigation was cut short as injection-site reactions were frequent and a small percentage of patients suffered anaphylaxis reactions[783]. These results do, however, suggest that the excipients in the drug formulation can have marked effects on pharmacokinetics of the drug.

Despite the potential adverse effects which can occur upon extended administration, in this study the SRB103 peptides will be injected daily using a $ZnCl_2$ diluent to improve the PK of both peptides. As previous work has shown liraglutide has a longer circulating half-life than the unformulated SRB103 dual agonists, a $ZnCl_2$ diluent was used to match of circulating half-lives of all peptides in the rodents.

5.1.2 Analysis of body composition

Ideally, the perfect anti-obesity incretin would reduce fat mass without any effect on lean mass. It has been speculated that chronic GCGR agonism would ultimately result in lean mass loss, as many glucagonoma patients display a distinctive reduction in lean mass[351,357]. Acute glucagon administration results in hypoaminoacidaemia, increased urea production and increases expression of enzymes critical for amino acid catabolisis in the hepatocyte[784,785]. Chronic GCGR agonism, therefore, also increases amino acid utilisation during gluconeogenesis. When the circulating pool of amino acids is depleted by enhanced gluconeogenesis rates, it is hypothesised that muscle and lean mass is catabolised to replenish the stock. Acute glucagon administration, however, would not cause a reduction in muscle mass as skeletal muscle does not express the GCGR. Also *Gcgr*$^{-/-}$ mice, and mice treated with a GCGR antagonist, are not greatly hypoglycaemic[353,354]; it has been suggested that it is actually cortisol that provides the gluconeogenic substrates, and glucagon increases the utility of these substrates[350,786]. Therefore, muscle wastage as a consequence of chronic glucagon agonism is not directly controlled by glucagon but is a secondary response to prolonged glucagon activity. The implications of chronic GCGR agonism in diabetic individuals, who would require muscle as a key insulin-sensitive tissue, would therefore be vitally important. This hasn't been investigated thoroughly in the context of dual GLP-1R/GCGR agonists. Consequently, measuring body composition, including lean and fat mass, after a chronic study including dual GLP-1R/GCGR agonists is important.

Anthropometry is the most basic method to analyse body composition, describing body mass, shape and size as well as approximations of adiposity[787]. Measurements include BMI, abdominal circumference and skinfolds. These basic metrics are useful for basic body composition analysis and can quickly allow a clinician, researcher or patient to broach the severity of obesity. However these measurements do not quantify amounts of different tissue types (fat, lean or water) and BMI has many basic flaws, including lack of accountability for highly muscular subjects, and a tendency for taller subjects to have a greater BMI than a shorter person with similar adiposity[788].

As anthropometry lacks translation into different body types, more in-depth investigative techniques are used. Dual-energy X-ray absorptiometry (DEXA) analyses body composition by evaluating the absorption of high- and low-energy X-rays. Absorption patterns are distinctive to tissue type, therefore allowing analysis of the whole body composition[789]. NMR, used in magnetic resonance imaging (MRI) scanning, similarly uses signature properties of fat, lean and water mass to evaluate whole body composition, however this is the less toxic analysis of hydrogen density and spin when exposed to a high power magnet[790]. In validation studies, DEXA displays tight precision and reproducibility of measurements, however the accuracy of the results are generally variable compared to chemical analysis for fat mass, and studies have shown it overestimates fat mass[791,792] and lean mass[791]. DEXA allows for the locality of fat mass to be observed which can be useful when dissecting visceral from s.c. fat. However, this comes at the cost of requiring the animal to be anaesthetised and exposed to more harmful X-rays. NMR is much less invasive and much safer, as it doesn't require anaesthetisation or harmful X-rays and takes less time than DEXA analysis[793]. Whilst it is shown to underestimate fat and lean mass[791], it is generally preferred for whole body composition for the reasons stated. Indeed, fat and lean mass can be chemically extracted in terminal studies, and provides the most accurate measure of fat and lean tissue in animal studies[791].

5.1.3 Acute versus chronic effects of biased incretin therapies

In the study to date, SRB103Gln3 has been shown to improve acute glucose homeostasis as seen in IPGTTs. Therapeutically, GLP-1R agonists and dual GLP-1R agonists are taken repeatedly to prolong weight loss and reduce hyperglycaemia, therefore increasing the duration of the investigation from acute to chronic studies is both important and interesting in establishing its potential as a therapeutic. As this study is the first to describe bias specifically with dual GLP-1R/GCGR agonists, this translation will be novel and potentially exciting.

One of the key therapeutic outcomes of dual GLP-1R/GCGR therapies in obese type 2 diabetic patients is remission of hyperglycaemia and glucose intolerance. Acutely, GCGR activation is linked to hepatic glycogenolysis and gluconeogenesis which can be counteracted by GLP-1R agonism. Indeed studies comparing GLP-1R mono-

agonists *versus* dual GLP-1R/GCGR agonists have displayed comparable acute (i.e. less than one day) [442,631] and chronic[436,437,442,729] glucoregulatory abilities in DIO mice, as well as in man[227,228], linked to an improvement in insulinotropic effects with the addition of GCGR activation to GLP-1R signalling. Alongside improvements in glycaemic control, addition of GCGR signalling also increases weight loss compared to GLP-1R signalling alone in humans[227,228]. Even though GLP-1R agonists have been shown to cause some weight loss by increasing energy expenditure, as shown by pair feeding studies[794], the primary mechanism of GLP-1R-mediated weight loss is reduced food intake. More recently it has been suggested that incretin therapy may lead to an improvement in NAFLD, one of the most prevalent liver diseases which is highly associated with obesity[795,796]. GLP-1R agonists have a proven ability in both mice[276,502,504] and humans[797-800] to reduce hepatic lipid content, separate from any body weight lowering abilities. However, incorporation of GCGR signalling exceeds the therapeutic potential of GLP-1R mono-agonists for the treatment of NAFLD beyond that of GLP-1R signalling alone[436,801,802]. Cumulatively, this highlights the exciting potential that dual agonism plays beyond simple acute glucose regulation improvements. The other therapeutic benefits elicited, such as increased weight loss, increased insulin secretion and improved hepatic outcomes in NAFLD likely all combine synergistically to create a more efficacious treatment.

With regards to bias at the GLP-1R, acute *versus* chronic effects are apparent. Three studies have investigated the implication of GLP-1R bias on acute and chronic metabolic parameters[276,502,504]. There was a greater divergence in anti-hyperglycaemic abilities of all the biased agonists compared to the balanced GLP-1R agonist, as studies progressed from acute to chronic daily injections. Interestingly, one biased agonist showed no insulinotropic effect[502], but improved HbA1c, whilst the other two displayed defined improvements in insulinotropic abilities after chronic administration[276,504]. Two of the biased agonists showed no effect at reducing body weight after chronic studies compared to the balanced agonist, however one biased agonist did elicit an effect[504]. Both biased agonists significantly improved hepatic steatosis scores, independent of overall weight loss, consistent with preclinical and clinical studies of GLP-1R agonism on liver steatosis[803,804]. These data suggest improved metabolic capabilities of biased GLP-1R agonists compared to unbiased equivalents.

As addition of GCGR signalling to GLP-1R signalling increases energy expenditure, and thus weight loss, and improves hepatic steatosis without deleterious effects on anti-hyperglycaemia, it is possible to envisage that creation of a biased dual agonist will incorporate the beneficial effects of bias and GCGR signalling into a superior therapy.

5.1.4 Aims

Having ascertained that SRB103Gln3 displays exciting therapeutic effects acutely, the aims of this chapter are to:

1) Examine the chronic effect of SRB103Gln3 on body weight, food intake and glycaemic control.
2) Explore how body composition may be affect by chronic administration of SRB103Gln3.

5.2 Results

5.2.1 SRB103Gln3 does not cause increased chronic weight loss compared to SRB103His3

Both SRB103 peptides were investigated to explore whether the biased SRB103Gln3 displayed altered therapeutic effects outside of acute anti-hyperglycaemia. To expand into chronic studies, randomised weight matched groups of DIO C57BL/6J mice received daily s.c. injections of either SRB103 peptides, the GLP-1R agonist liraglutide or vehicle at the onset of the dark phase to maximise drug effect. SRB103 peptides were reconstituted in a $ZnCl_2$-based diluent, developed from previous work in-house, which allows slow, prolonged release of peptide from a s.c. depot into the circulation. Two doses were used, which were up-titrated within the first week to prevent excessive weight loss in the first phase response (see section 2.15). IPGTTs were performed after two weeks when the peptide-treated groups were approximately weight matched, with body composition analysed within a couple of days of the IPGTT. Mean starting weights of the DIO mice were 38.6 ± 0.7g and 40.4 ± 1.0g for the 50nmol/kg and 20nmol/kg studies respectfully.

By the end of the high-dose study, there was no difference in body weight loss between the treatment peptides. Peptide treatment resulted in a significant reduction in body weight by the end of the study compared to vehicle treatment (Two-way ANOVA; $P<0.0001$). Groups treated with peptides at 50nmol/kg resulted in a weight loss of 7.0 ± 0.6g, 8.7 ± 0.7g and 8.9 ± 0.9g ($P>0.05$) for liraglutide, SRB103His3 and SRB103Gln3 respectively compared to final vehicle-treated mouse weights (Figure 5.1A). Observationally, mice were well-matched for weight loss between each treatment group until day 14, whereby liraglutide-treated mouse weights appeared to plateau whilst SRB103 peptide treatment caused sustained weight loss.

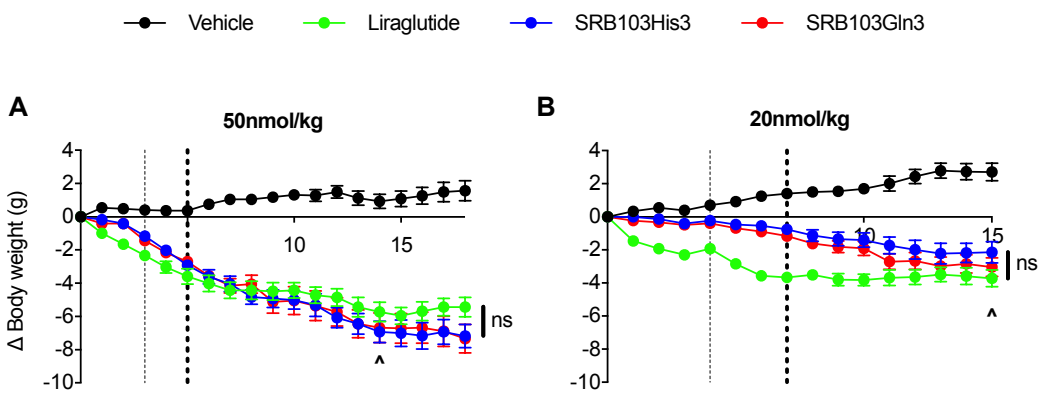

Figure 5.1 - Effect of chronic administration of SRB103 peptides and liraglutide on body weight in DIO mice

DIO mice were injected subcutaneously with SRB103His3 (blue), SRB103Gln3 (red), liraglutide (green) or vehicle (black) and body weight measured as indicated. Arrow denotes i.p. glucose tolerance test performed. (A) Mice initially received 16nmol/kg peptide, then 32nmol/kg (thin line) then finalised at 50nmol/kg (thick line) (n=10). (B) as for (A) but doses were 10, 16.7 and 20nmol/kg (n=10). Data presented as mean ± SEM. Data analysis performed using two-way ANOVA with Tukey post hoc test. Ns – not significant (P>0.05)

In the groups treated with peptides at 20nmol/kg, a final weight loss of 6.4 ± 0.5g, 4.9 ± 0.7g and 5.7 ± 0.6g was achieved for liraglutide, SRB103His3 and SRB103Gln3 treated mice respectively, of which there was no significant difference between either treatment (P>0.05) (Figure 5.1B). Similarly, there was a trend in the 20nmol/kg study for liraglutide treatment to eventually lead toward a plateau and a small average gain of weight by day seven, and here, the effect of SRB103 peptides also appeared to be plateauing toward the end of the study.

The differences in body weight were not significant between SRB103 peptides, despite SRB103Gln3 causing slightly greater weight loss in both studies compared to SRB103His3 (P>0.05). These data suggest that SRB103Gln3 does not have a greater effect on chronic weight loss compared to SRB103His3. At higher doses, both of the SRB103 peptides show a trend (however not significant) toward greater body weight loss than GLP-1R agonism alone, and an extended chronic study is required to confirm any difference in body weight reductions between treatments.

5.2.2 SRB103Gln3 does not decrease food intake chronically compared to SRB103His3

From the data presented in the previous section, SRB103Gln3 did not produce an improvement in weight loss compared to the balanced dual agonist SRB103His3, but both SRB103 peptides show a trend for greater weight loss compared to liraglutide when administered at high dose, which is lost at low dose. In acute studies, SRB103Gln3 tended to cause decreased food intake compared to SRB103His3 despite no statistical difference between the two. To observe if this trend translated after chronic administration, and to compare the food intake of both SRB103 compounds compared to the GLP-1R agonist liraglutide, food intake was measured alongside the body weight presented above.

In the high dose study, vehicle treated mice ate a total of 46.1 ± 2.0g over the study. Peptide treatment caused a significant reduction in food intake in all peptide treated groups with food intake of 32.5 ± 1.3 (Two-way ANOVA; $P<0.001$), 36.6 ± 1.4 ($P<0.01$) and 37.4 ± 0.8g ($P<0.01$) food eaten in liraglutide, SRB103His3 and SRB103Gln3 groups respectively (Figure 5.2A). There was no significant difference between SRB103His3 and SRB103Gln3 treated mice ($P>0.05$), however SRB103Gln3 treated mice ate significantly more than liraglutide treated mice ($P<0.05$).

In the lower dose study, vehicle treated mice ate a total of 43.9 ± 1.3g over the course of the study. Similar to the higher dose study, all peptide treatments caused a significant reduction in food intake with food intake of 32.3 ± 1.8 ($P<0.001$), 37.3 ± 1.5 ($P<0.05$) and 36.5 ± 1.6g ($P<0.01$) calculated in liraglutide, SRB103His3 and SRB103Gln3 groups respectively (Figure 5.2B). There was no significant difference in food intake between any of the treatment groups (all $P>0.05$).

These data collectively show that there is no food intake effect associated with SRB103Gln3 compared to SRB103His3, however there is a trend suggesting both dual agonist-treated groups ate more over the study than liraglutide treated mice in both studies.

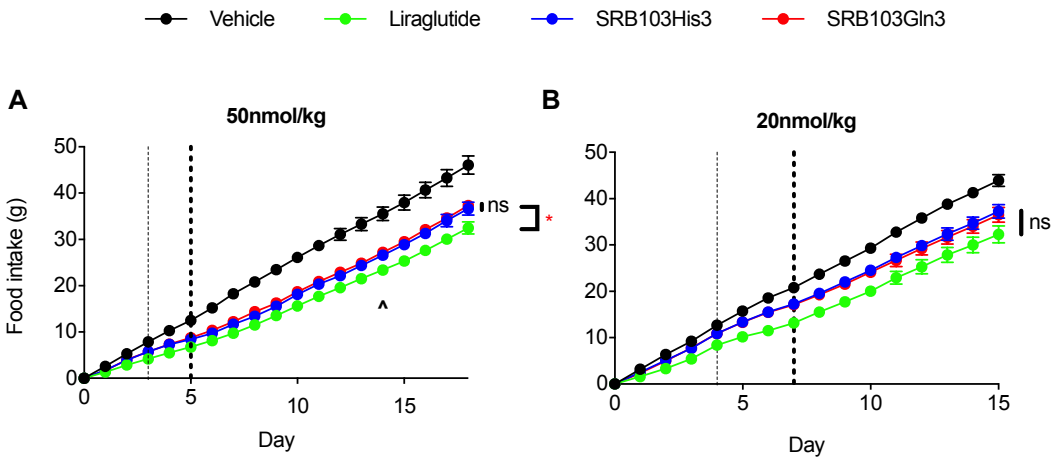

Figure 5.2 - Effect of chronic administration of SRB103 peptides or liraglutide on food intake in DIO mice

DIO C57BL/6J mice (6-8 months) were injected subcutaneously with SRB103His3 (blue), SRB103Gln3 (red), liraglutide (green) or vehicle (black) and food weight measured as indicated. (A) 50nmol/kg study (n=10). (B) 20nmol/kg study (n=10). Data presented as mean ± SEM. Data analysis performed using two-way ANOVA with Tukey post hoc test.; * P<0.05; ns – not significant (P>0.05).

5.2.3 SRB103Gln3 tends toward improved chronic anti-hyperglycaemia

In acute studies, SRB103Gln treatment displayed superior anti-hyperglycaemic effects compared to SRB103His3 over 4- and 8-hour peptide treatment times. Despite no effects observed between the two SRB103 peptides with regards to body weight or food intake, the effects on anti-hyperglycaemia were observed in a chronic setting. IPGTTs were performed after at least two weeks of peptide treatment and when average body weight of the peptide treated groups was matched to mitigate differences in body weight as a factor in the results (indicated by arrows in Figure 5.1). Injections were transitioned from the onset of the dark phase to just after initiation of the light phase, an hour after fasting, eight hours prior to the IPGTT. As with the acute studies, mice received an i.p. bolus of 2g/kg glucose after baseline blood glucose was measured and blood glucose was measured periodically afterwards.

After 50nmol/kg peptide administration (Figure 5.3A, top panel), all peptide caused a significant improvement in glucose tolerance compared to vehicle, as calculated by AUC (all P<0.0001). However, there was no significant difference between the liraglutide, SRB103His3 and SRB103Gln3 groups respectively (P>0.05) (Figure 5.3A, bottom panel).

In the lower dose study (Figure 5.3B top panel), a further reading was taken at 90 minutes post glucose administration. As before, all treatment groups displayed improved glycaemic control compared to vehicle treatment (all $P<0.0001$). However, liraglutide performed significantly worse in this study (Figure 5.3B, bottom panel) and the blood glucose measurements showing that the peak glucose reading at 20 minutes was very similar for both vehicle (25.7 ± 1.5mM) and liraglutide (24.8 ± 1.0mM). This is also interesting as 20nmol/kg liraglutide treatment resulted in a greater weight loss than 20nmol/kg SRB103Gln or SRB103His3, highlighting the superior anti-glycaemic effects of the SRB103 peptides separate from their abilities to induce weight loss. Here, SRB103Gln3 produced the greatest anti-hyperglycaemic effect, and was significantly better than liraglutide ($P<0.0001$) and tended towards greater anti-hyperglycaemia than SRB103His3, however this was not significant (AUCs of 0.95 ± 0.13M.min for SRB103His and 0.70 ± 0.05M.min for SRB103Gln3; $P>0.05$). SRB103His3 also displayed greater anti-hyperglycaemia than liraglutide ($P<0.01$).

This data suggests that the improvement in anti-hyperglycaemic effect is maintained in SRB103-treated mice when the dose is lowered, and there appears to be a trend toward SRB103Gln3 maintaining its superior anti-hyperglycaemic abilities compared to SRB103His3. Both SRB103 compounds outperform liraglutide at low dose, but all peptides are equally efficacious when administered at a higher dose. This suggests the subtle differences in anti-hyperglycaemic abilities between all compounds which are evident in the 20nmol/kg study are masked in the 50nmol/kg study.

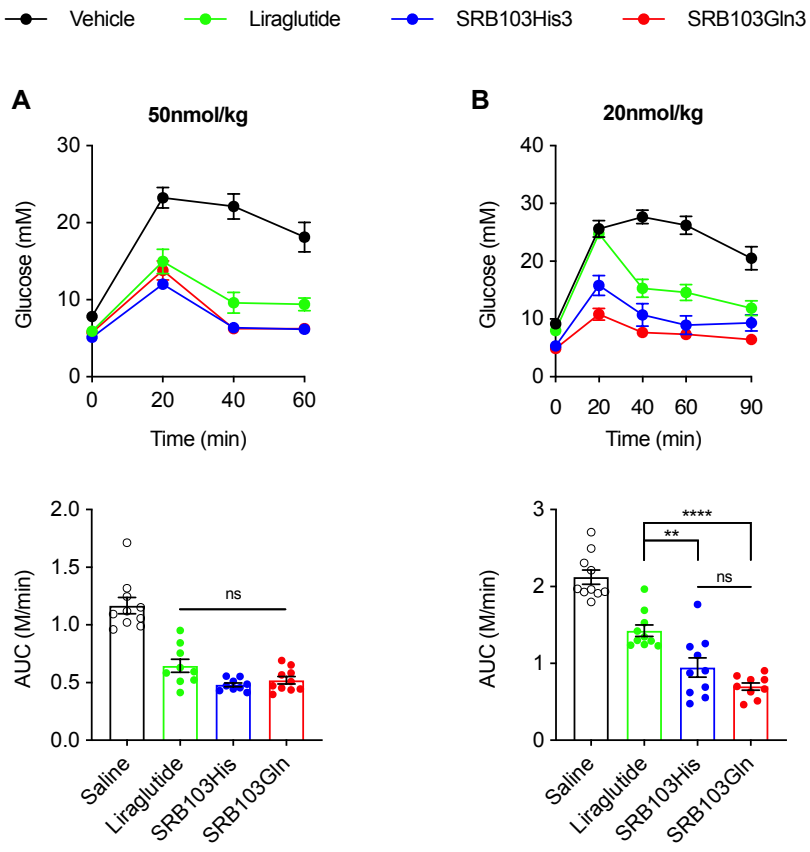

Figure 5.3 - Effect of chronic administration of SRB103 peptides or liraglutide on glucose tolerance in DIO mice

DIO C57BL/6J mice (6-8 months) were fasted an hour before s.c. injection of SRB103His3 (blue), SRB103Gln3 (red), liraglutide (green) or vehicle (black). Baseline blood glucose levels were taken via venesection 8 hours later before 2g/kg glucose was injected via the i.p. route. (A) Effect of 50nmol/kg peptide administration on blood glucose concentrations (top) with concomitant area under curve (AUC) calculation (bottom) (n=10). (B) as for (A) but after 20nmol/kg peptide administration (n=9-10). Data presented as mean ± SEM. Statistical analysis performed by one-way ANOVA with Tukey post hoc test. *** P<0.001; ** P<0.01; ns – not significant (P>0.05).

5.2.4 SRB103Gln3 has no differential effect on body composition compared to SRB103His3

In keeping with reduced body weight, the composition of lean and fat mass can differentially change with chronic administration of GLP-1R mono-agonists or dual GLP-1R/GCGR agonists. To investigate how chronic treatment of the SRB103 peptides and liraglutide affected the body composition of DIO mice throughout the chronic study, whole body composition analysis was performed using MRI (EchoMRI, UK), allowing for non-invasive quantification of fat, lean and water mass at different timepoints. This was performed the day before the IPGTT and at the end of the 50nmol/kg study, and then at the end of the 20nmol/kg study.

By the end of the 50nmol/kg study, mice treated with either SRB103His3 or SRB103Gln3 had lost a similar percentage of body fat mass, however SRB103Gln3 treatment caused a decrease compared to liraglutide treatment (two-way ANOVA; P<0.05) (Figure 5.4A, left panel). SRB103Gln3 treatment also resulted in the greatest lean mass loss out of all peptide groups (P<0.05 compared to vehicle), however all peptide treatments caused a similar reduction in lean mass. Saline treated mice also lost lean mass, perhaps explaining why only SRB103Gln3 displayed a significant reduction (Figure 5.4A, middle panel). There was no statistical difference in water loss between any group by the end of the study (P>0.05 for all) (Figure 5.4A, right panel).

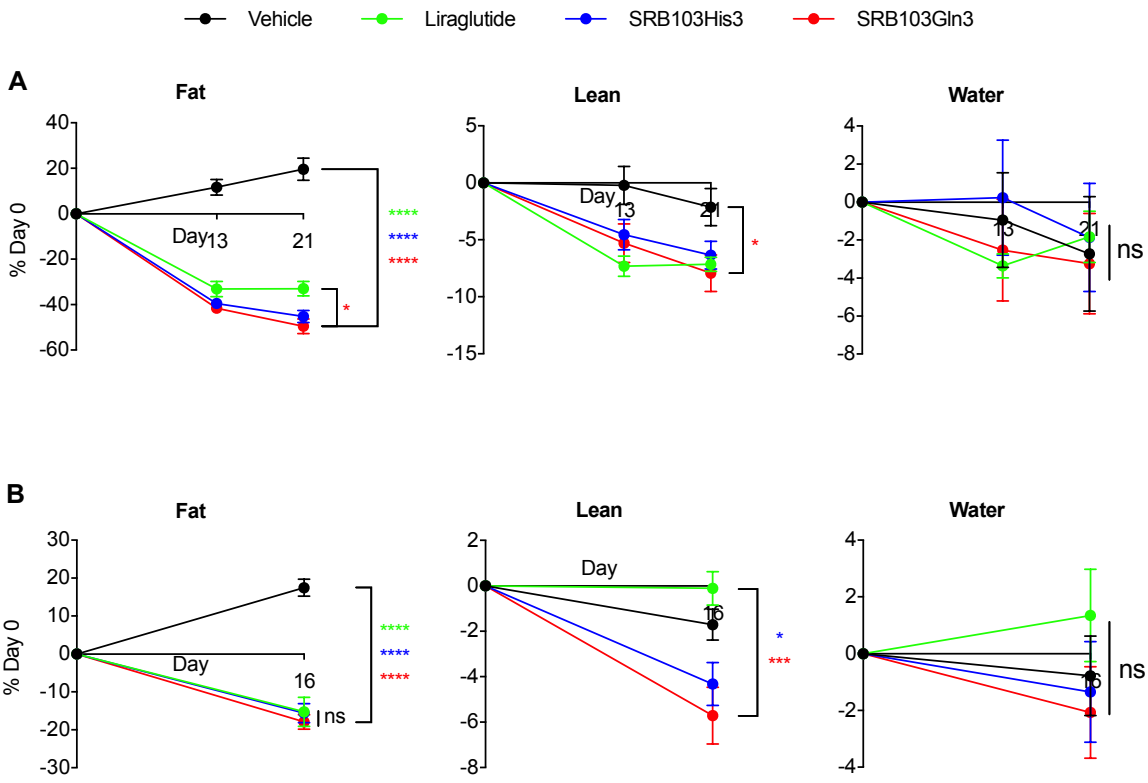

Figure 5.4 - Effect of chronic administration of SRB103 peptides or liraglutide on body composition

DIO C57BL/6J mice (6-8 months) had body fat mass (left), lean mass (centre) and body water mass (right) measured by MRI at the day indicated. (A) 50nmol/kg study where measurements were taken 13 days (day before the IPGTT) and 21 days after treatment commenced (n=10). (B) 20nmol/kg study where measurements were taken on 15 days (day after IPGTT) after treatment commenced (n=9/10). Data presented as mean ± SEM. Statistical analysis performed by two-way ANOVA (A) or one-way ANOVA (B) with Tukey post hoc test.

Data from the 20nmol/kg study shows that mice lose a statistically similar proportion of body fat mass from day zero after liraglutide, SRB103His3 and SRB103Gln3 treatment respectively (one-way ANOVA; P>0.05) (Figure 5.4B, left panel). This fat

mass loss is statistically greater than saline-treated mice, who put on 17.5 ± 2.3% body fat mass from day zero (all $P<0.0001$). Interestingly, both SRB103 treatments cause a similar significant reduction in lean mass as well, however it is greater with SRB103Gln3 treatment, with SRB103His3 reducing lean mass by 4.3 ± 0.9 % and SRB103Gln3 by 5.7 ± 1.3% ($P>0.05$) (Figure 5.4B, middle panel). Both of these are, however, significantly greater reductions than for liraglutide treatment ($P<0.05$ for SRB103His3, $P<0.001$ for SRB103Gln3). Again, there was no effect of changes in total water content between saline and peptide treatment by the end of the study ($P>0.05$) (Figure 5.4B, right panel).

In summary, data from the body composition analysis reveals that both SRB103 peptides are equally effective throughout both studies at reducing both fat mass and lean mass. There is no statistical difference between the SRB103His3 or SRB103Gln3, however SRB103Gln3 tends to result in a marginally greater fat and lean mass loss. Liraglutide in both studies is cumulatively not as effective as the SRB103 peptides, with less fat mass loss and lean mass loss in both studies.

5.2.5 SRB103Gln3 causes greater food intake reduction in lean rats

The data above suggests that SRB103Gln3 treatment does not exhibit significant body weight, body composition or food intake effects at either 50nmol/kg (high dose) or 20nmol/kg (medium dose) when compared to SRB103His3. Species-specific effects were further investigated, as pharmacological profiling had not been performed in mouse receptors to confirm an equipotent cAMP response, as was seen in human receptors. Rats were therefore used as a second species to look at daily body weight and food intake effects of SRB103Gln3, SRB103His and liraglutide. Doses of SRB103 peptide were calculated from previous studies as being “low-dose” (3nmol/kg), “medium dose” (6nmol/kg) and “high-dose” (12nmol/kg). Liraglutide was tested at 6 and 12 nmol/kg. Rats were injected subcutaneously with SRB103 peptide made up in $ZnCl_2$ diluent to sustain circulating levels.

At 3nmol/kg (Figure 5.5A), both SRB103 compounds were equally as ineffective at causing weight loss or food intake reduction. Vehicle-treated rats ate 87.1 ± 1.1g of food and increased body weight by 14.7 ± 1.3g whilst those on 3nmol/kg of

SRB103His3 and SRB103Gln3 ate a statistically similar weight of food and put on a similar amount of weight (two-way ANOVA; both P>0.05 with respect to vehicle and each other).

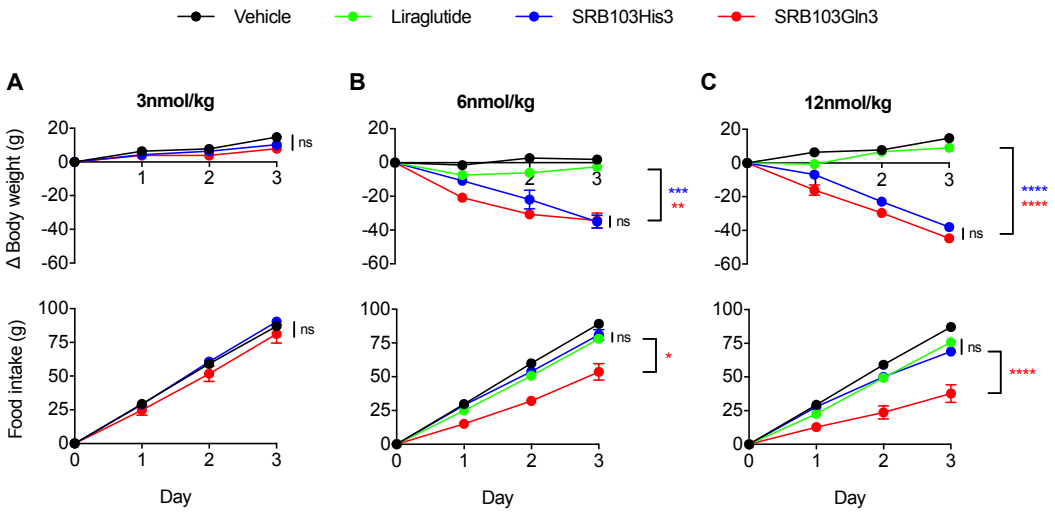

Figure 5.5 - Effect of chronic administration of SRB103 peptides or liraglutide on body weight and food intake in rats

Male Wistar rats (3 months) fed on standard diet received daily injections of liraglutide (green), SRB103His3 (blue), SRB103Gln3 (red) or vehicle (black) by the s.c. route and had body weight and food intake measured. (A) 3nmol/kg (n=6/7). (B) as for (A) but at 6nmol/kg and with liraglutide (n=6/7). (C) as of (B) but at 12nmol/kg (n=6/7). Data presented as mean ± SEM. Statistical analysis performed by two-way ANOVA with Tukey post hoc test. **** P<0.0001; *** P<0.001; ** P<0.01; * P<0.05; ns – not significant P>0.05.

An anorectic effect is evident at the medium dose (Figure 5.5B), where SRB103Gln3 caused a significant reduction in food intake compared to SRB103His3 (Two-way ANOVA; P<0.05). However, this did not translate into increased weight loss (P>0.05). Interestingly, the food intake of SRB103His3-treated rats was similar to 6nmol/kg liraglutide-treated mice (P>0.05). Both SRB103Gln3 and SRB103His3 at 6nmol/kg caused a significantly greater weight loss than 6nmol/kg liraglutide (P<0.001 for SRB103His and P<0.01 for SRB103Gln3).

Finally, at the highest dose tested (12nmol/kg; Figure 5.5C), the anorectic ability of SRB103Gln3 was even more evident. Here, SRB103Gln3 treatment almost halved the food intake compared to SRB103His3 over the three days (P<0.0001) as well as liraglutide (P<0.0001). Again, SRB103His3 treatment caused similar food intake reductions as liraglutide (P>0.05). Despite SRB103Gln3 causing an increase in weight loss compared to SRB103His3 (44.7 ± 2.5 *versus* 38.0 ± 2.1g respectively) this was

not statistically significant (P>0.05). These were both statistically greater than liraglutide (P<0.0001). Surprisingly liraglutide treatment did not reduce food intake or body weight significantly at 12nmol/kg compared to vehicle in this study (P>0.05 *versus* vehicle for both final food intake and body weight).

These data demonstrate a difference in repeat-dosing effects between species, where in rat, SRB103Gln3 causes large and sustained anorectic effects compared to SRB103His3, without significant effects on body weight. Both dual agonists appear to be much more successful at reducing body weight at medium to high doses, whereas liraglutide appears to display insignificant effects with regards to both parameters compared to vehicle.

5.3 Discussion

The aim of this chapter was to delineate whether there was any therapeutic advantage to using SRB103Gln3, a dual GLP-1R/GCGR agonist with low β-arrestin efficacy, over a balanced agonist in a chronic *in vivo* setting. Acutely, SRB103Gln3 appeared to demonstrate greater anti-hyperglycaemic effects after prolonged administration in lean and obese mice, theoretically down to both $G\alpha_s$ bias at the GLP-1R allowing prolonged stimulation of the GLP-1R, and partial agonism at the GCGR reducing acute hyperglycaemia associated with GCGR activation. Cumulatively, this creates a more anti-hyperglycaemic phenotype. SRB103Gln3 appeared not to have any effect on satiety acutely, in keeping with GLP-1R $G\alpha_s$ bias[276,502]. Therefore, two chronic studies were performed on DIO mice to investigate the wider therapeutic ability of the SRB103 peptides on body weight change, food intake, glycaemic control and body composition in a disease model. DIO mice were used in these studies as they encompass the environmental aspect of obesity, which is greatly-linked to type-2 diabetes in humans[633]. Polygenic models are more expensive and have physiologies distinct from those of an obese, diabetic mouse[640] therefore were not considered in these initial chronic studies. DIO mice were kept on HFD for three months before the chronic studies began, allowing their body weight to increase to approximately 40g on average and induce a more diabetic phenotype.

One of the greatest positives of this chronic study is the inclusion of the GLP-1R agonist liraglutide, which allowed the contribution of GCGR to be investigated as well as bias. Some chronic or human studies investigating dual agonists lack a unimolecular agonist as a comparator, which can leave comparisons of dual *versus* mono-agonist therapies unavailable.

The main results from this chapter are as follows:

1) There is no difference in body weight, food intake, glycaemic control or body composition between mice chronically treated with SRB103Gln3 or SRB103His3.
2) Both SRB103 agonists appeared to display greater effects on chronic anti-hyperglycaemia than the GLP-1R agonist liraglutide given at a low dose, without significant changes to body weight and food intake.

3) SRB103Gln3 causes large decreases in food intake in rats compared to SRB103His3, yet both cause a similar amount of weight loss.

5.3.1 SRB103Gln3 does not increase body weight loss or decrease food intake chronically in mice versus SRB103His3

In the chronic studies, DIO mice were injected with a daily s.c. injection of SRB103His3, SRB103Gln3, liraglutide or vehicle, at a high dose (50nmol/kg) or medium dose (20nmol/kg). Doses had to be increased from the acute studies (10nmol/kg) to account for a prolonged 24 hour effect, as opposed to the acute studies which only required a maximum of an 8 hour effect. Here, there was no difference between SRB103Gln3 and SRB103His3, suggesting there are no beneficial effects on body weight decrease by reducing β-arrestin efficacy at both the GLP-1R and GCGR with the dual agonists. By the end of both studies, mice treated with SRB103Gln3 or SRB103His3 had both lost the same amount of weight, with a similar reduction in food intake (Figure 5.1, 5.2). Both SRB103 peptides lost the same amount of weight as liraglutide treatment, however liraglutide-treated mice weights were beginning to plateau towards the end of the study after a large initial weight loss, and liraglutide-treated mice ate less in both studies. This suggests two things. Firstly, it suggests that there are compensatory mechanisms occurring with liraglutide treatment, as mice become metabolically adapted to constant GLP-1R agonism. This adaptation is distinct to that of the satiation pathway, as in both studies, liraglutide treatment maintained a reduced food intake compared to vehicle and the SRB103 peptides (Figure 5.2). Interestingly, OXM has been shown to reduce food intake in mice less than Ex4, attributable to reduced gastric emptying rate elicited by Ex4[805]. The data presented in the present study replicates this finding and suggests potentially beneficial effects of dual agonists on gastric emptying rates, which may account for some of the GLP-1R agonist-mediated gastrointestinal effects[806].

Secondly, as SRB103 peptides are on a trajectory of greater weight loss in the high-dose study, it highlights the enhanced energy expenditure effect of GCGR agonism and the importance of GCGR-mediated energy expenditure in a maintained weight loss. The GCGR is well regarded as being critical for energy expenditure in dual GLP-1R/GCGR agonists[227,442,729], with its implications on increased weight loss[437,729].

Whilst my observations are not novel, as the efficacy of dual agonists has been extensively examined before, it perhaps adds further evidence that the mechanism by which SRB103Gln3 exhibits its therapeutic effects is relating to bias at the GLP-1R and not the GCGR. SRB103Gln3 displays improved anti-hyperglycaemic abilities, but no effect on body weight, compared to its unbiased comparator. This therapeutic phenotype is remarkably similar to the cAMP-biased GLP-1R agonist Ex-Phe1[276]. Whilst similar doses of chronic liraglutide have been associated with relatively small increases in cold-induced energy expenditure in DIO previously[794], the extent of energy expenditure associated with GLP-1R signalling alone is minimal in comparison to the integration of GCGR signalling as well.

Intriguingly, the extent of food intake reduction is the same in both the high- and low-dose studies. In both studies, regardless of dose, administration of either SRB103 peptide resulted in a final food intake of between 36.5 – 37.3g, whilst liraglutide treatment resulted in an average food intake of 32.5g for the high dose study and 32.3g for the low-dose study. This suggests that the anorectic effect for all peptides may be at their maximum capacity even at lower doses. In spite of the similar food intake between the two studies, SRB103Gln3-treated mice lose an extra 4.2g of body weight (compared to vehicle) when they switch to the higher dose, and SRB103His3 lose an extra 3.8g. Meanwhile, liraglutide-treated mice only lose an extra 0.6g compared to vehicle when given the higher dose. This further highlights the impact of GCGR-mediated weight loss. It also suggests that the receptor-response coupling required to affect food intake is less than the coupling required to initiate energy expenditure, i.e. a higher concentration of ligand is required to initiate pathways to instigate energy expenditure. This could relate to the phenomena of “tissue bias” whereby a ligand can differentially activate signalling pathways in different tissues based on the tissue “coupling ability” to that receptor (such as relative expression of $G\alpha_s$ or β-arrestin). This is an emerging field of pharmacology, and requires greater delineation to understand how drugs acting at the same receptor can elicit different responses at different tissues.

The drawback of repeated injections without extensive PK testing is this study does not take potential drug accumulation into account, in either mice or rats. Here, it is assumed, based on previous work in-house with similar peptides, that there is a steady

state blood concentration of peptide over the period of study. Many GLP-1R agonists induce nausea *in vivo*, which results in altered feeding behaviours in mice and rats[326]. Therefore, if time had permitted, it would have been advantageous to perform a PK study after chronic administration to investigate whether peptide was accumulating in the system after repeated injections, and secondly perform condition taste avoidance to test whether the repeated dosing was causing nausea. A PK study would also confirm how the circulating levels of SRB103 peptide and liraglutide differed throughout the study. The physico-chemical make-up between liraglutide and SRB103 peptides likely mean the PK parameters of absorption, distribution and metabolism are distinct from one another, which was not accounted for in this study. The relative potency of liraglutide or the SRB103 peptides to the GLP-1R were also not considered, which may explain differences in food intake, however the ability to translate such *in vitro* pharmacology to chronic *in vivo* studies is rarely possible.

As with the acute studies, it would be interesting to repeat the chronic studies in a number of ways to better understand the pharmacological effects of each peptide investigated here. Firstly, matching by body weight loss or food intake will allow for the role of each peptide to be examined for each parameter, without the interference of the other. It would also be interesting to perform these with additional groups containing antagonists at either receptor to further investigate the role that each receptor plays in the pharmacology of the individual SRB103 peptides. Transgenic mice have altered phenotypes, such as *Gcgr*$^{-/-}$ mice showing a trend for altered metabolic profiles and body composition and *Glp1r*$^{-/-}$ displaying altered body composition and glucose intolerance[354,685,807-809]. Therefore, using antagonists to inhibit receptors throughout a study allows for the effects of GLP-1R and GCGR signalling to be examined in the context of chronic SRB103His3 or SRB103Gln3 treatment whilst mitigating the altered phenotypes discussed with transgenic mice. However, as noted in chapter 4, long-acting antagonists at these receptors have yet been validated.

5.3.2 SRB103Gln3 has much greater anti-obesity effect in rats than mice

Results for the chronic studies were confirmed in lean Wistar rats. Previous work in-house to determine the potency of the peptides to the rat receptors and the PK profile

of similar peptides in rats generated the three doses to use in the study. In rats, SRB103Gln3 reduced food intake by between a third and a half at 6nmol/kg and 12nmol/kg without significant affects to body weight loss compared to SRB103His3 (Figure 5.5). This suggests that SRB103His3 is able to induce greater weight loss through increased energy expenditure.

In the previous section, the role of the GCGR was implicated as being vital for energy expenditure-induced weight loss in mice. One suggestion for the mechanism of weight loss in rats is that SRB103His3 is a full agonist at the GCGR, which results in increase GCGR-mediated energy expenditure and ultimately drive weight loss without the need for a lack in food intake. In line with this theory, as SRB103Gln3 is a low-efficacy GCGR agonist, it may not be able to couple sufficiently to GCGR pathways in rats, which regulate energy expenditure, therefore it has little effect on energy expenditure. Similarly, as SRB103Gln3 displays reduced efficacy for β-arrestin-2 recruitment at the GLP-1R, it stands to reason that prolonging GLP-1R activity (by negating β-arrestin-induced internalisation) would compensate the lack of GCGR-mediated weight loss by severely reducing food intake and concomitant weight loss. As this phenotype is witnessed in the rat studies, it further supports the NanoBiT assay results and questions the cAMP and PathHunter assay results. Other potent GLP-1R agonists show a similar initial phenotype as SRB103Gln3, with great reductions in food intake and body weight in rats[810-812], however food intake and body weight usually plateaus or even compensates to that of vehicle treatment, likely due to desensitisation. Not only does SRB103Gln3 likely elicit reduced GLP-1R desensitisation, it also signals at the GCGR to prolong and maintain the initial effects.

However, this does not appear to be the case in mice, where both SRB103 peptides resulted in similar weight loss (Figure 5.1). This may be explained by the phenomena of “tissue-bias”, but in this case, the bias is between the same tissue of different species. Indeed, species-specific differences have been observed in response to the same stimulus[813,814]. In mice, the $G\alpha_s$ response produced by high-dose SRB103Gln3 could be sufficient to pass the threshold of initiating GCGR-mediated energy expenditure, whilst this might not be the case in rats. However, this requires knowledge of how both SRB103 peptides signal at both the rat and mouse GLP-1R

and GCGR homologues, and understanding of how the different signalling profiles match the observed phenotype *in vivo*.

Liraglutide displayed a muted therapeutic effect in this study. It's only effect in the rat studies was the mild reduction in food intake at 12nmol/kg (saline - 87.1 ± 1.1g; liraglutide 75.8 ± 2.6g; $P<0.05$) however this did not reduce body weight efficiently. Interestingly liraglutide appears to have elevated effects in obese rodents and little effect in lean rodent[815]. The rats used in this study were fed on a standard chow diet, and despite a mean average weight of 472g, were not considered an obese rat model. This could explain why it is so effective in the DIO mouse studies, and ineffective in the rat study. The rats used were not obese, and therefore repeating on a DIO cohort of rats would be an interesting future study to perform. However, the HFD pellets are friable, meaning that it can be difficult to precisely measure true food intake in an HFD study. Considering the food intake in vehicle-treated rats was 25g per day, the friable nature of the diet could significantly affect the accuracy of food intake.

Further studies in rats could be performed, in the presence of antagonists, to support the suggestions made above. As discussed in the previous chapter, antagonists capable of inhibiting receptor signalling *in vivo* have not been validated in this work, and therefore further study would have to go into ensuring antagonists to the receptors were potent, effective and pharmacokinetically viable.

5.3.3 SRB103Gln3 and SRB103His3 are equally anti-hyperglycaemic in chronic studies

The acute anti-hyperglycaemic properties that SRB103Gln3 displays makes it an attractive candidate for obese diabetics, as it produces an improvement in glucose tolerance through increased insulin secretion, whilst having no effect on food intake, which is associated with nauseating side effects. In these chronic studies, IPGTTs were performed to confirm whether the improved anti-hyperglycaemic phenotype was maintained after chronic administration. In the higher dose study, all peptides were equally anti-hyperglycaemic eight hours after injection (Figure 5.3A) whereas in the lower dose study, the efficacy of liraglutide reduced whilst both SRB103 peptides remained equally as anti-hyperglycaemic, however the trend observed acutely for

SRB103Gln3 to display greater anti-hyperglycaemia compared to SRB103His3 was replicated (Figure 5.3B).

Any changes in body weight or food intake, which may influence glucose tolerance between either SRB103 peptides are mitigated by the fact they ate the same amount and lost the same amount of weight. The contribution of body weight is an important factor which leads to improved anti-diabetic phenotype witnessed in many post-bypass patients[816,817], but as previous acute studies negate differences in food intake, body weight or composition, SRB103Gln3 clearly exerts its effects outside of this remit. One suggestion as to why the divergence between the two SRB103 peptides was lost after chronic injections could be that the $ZnCl_2$ diluent affects the pharmacology of SRB103His3. However, this was quashed by performing separate acute IPGTTs with the SRB103 peptides made up in either 0.9% saline (used in the acute studies) or $ZnCl_2$ diluent (data not shown). This showed that SRB103His3 did not become more anti-hyperglycaemic when injected s.c. in the $ZnCl_2$ diluent compared to i.p. in saline, suggesting that administration route and diluent did not have an effect on the IPGTT results from the chronic study. Another reason why SRB103His3 and SRB103Gln3 show statistically similar anti-hyperglycaemia in the chronic studies could relate to drug accumulation, as discussed in section 5.3.1. Acute anti-hyperglycaemic effects of SRB103Gln3 were observed in relatively low circulating concentrations such as 10nmol/kg used in this work. When acute drug concentrations were as high as 100nmol/kg, divergence in anti-hyperglycaemia between the SRB103 peptides was lost as the maximum capability of the insulin secretion system is achieved (data not shown). Therefore, it is possible that a similar phenomenon is occurring in both of the chronic studies, whereby drug accumulation results in maximal insulin secretion in both SRB103 peptide groups and therefore divergences are not possible.

Further investigation into this should be made, and a number of studies can be done. Firstly, PK studies should be performed to investigate the circulating concentration of peptide after 14 days of daily s.c. injections. HPLC analysis of DPP-IV peptidase activity suggested no significant difference between the SRB103 peptides, however any cumulative effects of repeated dosage weren't investigated. This could help to understand if there is accumulation of drug in the system, which could explain the lack of difference between SRB103His3 and SRB103Gln3 in the IPGTT. This could lead to

a repeated study where the doses are reduced which may prise apart the chronic glucoregulatory abilities of the two peptides. It would also be interesting to measure insulin secretion during chronic SRB103His3 and SRB103Gln3 administration, using the HTRF assay as seen previously in this work. Acutely, SRB103Gln3 induced a greater insulin secretion at prolonged treatment time points (Figure 4.2C) which was the likely reason for improved acute glucoregulatory abilities. Perhaps chronic treatment of SRB103 peptides causes equivalent insulin secretion, which would result in similar IPGTT anti-hyperglycaemic abilities.

Regardless of this, a treatment for obesity *and* diabetes must account for both weight loss and glycaemic control. Whilst reducing the dose may result in SRB103Gln3 displaying greater anti-hyperglycaemia compared to SRB103His3, if this comes at the cost of minimal body weight loss, then there isn't a therapeutic advantage in utilising bias for the dual GLP-1R/GCGR agonists discussed here. Alternatively, minimal weight loss with associated improved anti-hyperglycaemia could switch the targeting of SRB103Gln3 toward an overweight, not severely obese, individual with severe T2DM.

5.3.4 SRB103 compounds induce greater lean mass loss than liraglutide at higher doses

One of the novel and interesting parts of this work is additional MRI analysis of body composition after the chronic study. This provides a deeper understanding of where body mass loss is occurring, which can begin to explain the results for the chronic studies. Due to the relatively invasive nature of this procedure, mice were only tested for body composition around the time of the IPGTT and at the end of the study. For the high-dose study, the GTT was performed on day 14 so an MRI was performed the day before on day 13, and the study ended seven days after the GTT on day 21, which is when the final MRI was performed. However, body weight and food intake were not recorded between day 18 and the end of the study on day 21, therefore it is unknown how divergent the average weight of the different treatment groups were. In the second study, to reduce any impact that stress of the MRI could have in the IPGTT results, the 20nmol/kg study the MRI was performed after the GTT on day 15, which was also

the day the study terminated. This lack of protocol linearity makes comparisons between the two studies more tenuous.

In the 50nmol/kg study, by the end of weight recordings on day 18 there was a trend suggesting both of the SRB103 peptides had caused a greater weight loss than liraglutide, however this was not significant (Figure 5.1). Three days later, MRI data showed that SRB103Gln3 peptide treatment resulted in a significant reduction in body fat mass compared to liraglutide treatment (49.5 ± 3.2 *versus* 32.9 ± 3.2% respectively). SRB103His3 matched the trend of SRB103Gln3 for fat mass loss (45.2 ± 2.7%) however the difference between SRB103His3 and liraglutide was not significant. All peptide treatment showed a similar lean mass loss of between 6-8% from day 0, and no significant effects in water loss. In the lower dose study at 20nmol/kg, the reverse is true; that is, all peptide treatments lose a statistically similar percentage of body fat (15-18%), however both SRB103 peptides cause a significant reduction in lean mass of 4.3 ± 0.9 and 5.7 ± 1.3% for SRB103His3 and SRB103Gln3 respectively, whilst liraglutide treatment results in essentially zero lean mass loss (0.1 ± 0.7% lost).

Cumulatively, these data suggest that there are two separate mechanisms which are causing weight loss in the separate studies. Increasing the dose in the high-dose study will increase the likelihood of surpassing the threshold of developing nausea. As nausea in mice manifests in a reduction in locomotion, the high dose liraglutide treatment could result in a sedentary phenotype in mice. At lower levels such as 20nmol/kg, the likelihood of remaining within the therapeutic window is increased, thus reducing the likelihood of nausea. Therefore, lean mass is not lost in the liraglutide group as they are more active. This is, however, a speculative theory. This theory does, however, agree with previous studies investigating body composition relating to incretins and exercise. Other studies investigating body composition after chronic GLP-1R agonist treatment have witnessed similar results to the 20nmol/kg liraglutide results, that is fat mass reduces whilst lean mass remains[818]. This likely occurs with liraglutide as well, as chronic liraglutide treatment at concentrations of 10 or 30nmol/kg results in activation of BAT in DIO mice, resulting in reduced adiposity with minor effects on lean mass[794]. GLP-1R agonism has even been shown to ameliorate muscle atrophy, suggesting primary GLP-1 signalling is not a cause for lean mass loss[819]. This

suggests chronic GLP-1R agonists could reduce adiposity without diminishing lean mass. It would be interesting to repeat and include analysis of circulating amino acid levels and qPCR of key genes involved in gluconeogenesis and lipolysis to firstly confirm the interesting body composition results and investigate how the mechanisms behind GLP-1R activation, amino acid flux and reduced adiposity are linked.

MRI is one of the two predominantly used methods for analysing total body composition, the other method being DEXA. In this study, MRI was used as it is a rapid, less invasive process which does not require anaesthetisation[793]. However, comparator studies have noted that MRI tends to underestimate fat and lean mass[791]. Therefore, it would be useful to consolidate the body composition data collected from the MRI with chemical analysis of fat and lean mass, which provides a more accurate representation of body composition.

In conclusion, this chapter has further elucidated the *in vivo* effects of SRB103Gln3 beyond its superior acute glucoregulatory response. Whilst it tended towards increasing greater weight loss, reducing food intake, maintaining improved glucose tolerance and increasing fat and lean mass loss *versus* SRB103His3 in mice, these results were statistically not significant. In rats, there is an immediate and sustained suppression of food intake concomitant, however no difference in weight loss when treated with SRB103Gln3 compared to SRB103His3. Further investigation is required to confirm the results presented above, and to delve into mechanisms explaining the results above.

6 General Discussion

Obesity is a global epidemic, with 650 million adults worldwide estimated to be obese, and a further 1.3 billion overweight[78]. The rate of prevalence is rapidly rising and is a global medical concern[78]. Obesity is closely linked to co-morbidities including type 2 diabetes mellitus (T2DM), and estimates suggest 90% of T2DM patients are obese or overweight[8]. T2DM is defined by chronic hyperglycaemia, stemming from a reduced ability to secrete and utilise insulin to sequester rises in circulating glucose, and is linked to an increased risk of myocardial infarction, stroke, cardiovascular disease and neuropathy[8]. It is estimated to affect 500 million people and is in the top ten leading causes of early mortality, highlighting the critical nature of tackling obesity-induced T2DM[6].

Roux-en-Y gastric bypass (RYGB) surgery is currently the most successful therapeutic intervention for the treatment of obesity and T2DM. Human longitudinal studies show weight loss can reach up to 32% three years post-surgery and 28% seven years post-surgery[820,821], and near total remission of diabetic symptoms is seen seven years after RYGB surgery specifically[821]. RYGB is associated with an increase in circulating incretins, which aid in postprandial satiation, digestion and sequestration of carbohydrates and fats[220,221]. These incretins include GLP-1 and OXM, both of which act as agonists at the GLP-1R to augment glucose-stimulated insulin secretion (GSIS), increase insulin sensitivity and improve pancreatic β cell function[220,221]. However, bariatric surgery is an invasive procedure and is associated with an unacceptable surgery-related ten year mortality rate of approximately 1.5%[822,823], as well as significant morbidity. It could be considered a costly procedure both for the patient and healthcare provider, therefore alternative methods are required.

There is an unmet need for obesity and diabetes treatments which are both highly efficacious and have an acceptable side effect profile. There are a number of pharmacotherapies available to treat T2DM, including insulin, sulphonylureas and TZDs. However, all of these treatments can in fact lead to weight gain[146,179,180,196]. Targeting the incretin pathway is another approach, either using DPP-IV inhibitors (thus blocking endogenous incretin degradation) or with stable GLP-1R agonists, to improve GSIS[292]. Moreover, DPP-IV inhibitors are weight neutral, whilst GLP-1R agonists produce weight loss alongside their direct glycaemic effects. Therefore, targeting the incretin pathway carries advantages over standard diabetes treatments

to treat the obese diabetic patient population. Despite this, GLP-1R agonists are also associated with side effects, including nausea and GI effects, and dose escalation studies suggest optimal therapeutic efficacy has yet to be achieved due to these dose-limiting side effects[460]. Therefore, there is a clear opportunity to optimise incretin-based drugs to increase their therapeutic potential.

One suggested route of optimisation is introducing a secondary metabolic receptor target for the ligand, such as the GCGR. This could elicit numerous theoretical advantages, including reduced amounts of GLP-1R activation, thereby minimising GLP-1R-mediated side effects, as well as improved insulinotropic and glucoregulatory capacity and increased weight loss[824]. Whilst glucagon is associated with acute hyperglycaemia driven by hepatic glycogenolysis and gluconeogenesis, co-stimulation with GLP-1 has been shown to suppress hyperglycaemia[227,228,381]. Additionally, prolonged GCGR stimulation leads to increased weight loss through increased energy expenditure, improved insulin secretion and sensitivity[227,228,394]. Therefore, dual GLP-1R/GCGR agonists will likely be synergistic in their ability to combat obesity-induced T2DM. Indeed, rodent studies have highlighted that dual GLP-1R/GCGR agonists display superior efficacy for weight loss and glucose regulation compared to GLP-1R agonists alone[436,437,825], but these ligands still result in nauseating and GI-related side effects. This suggest that, whilst improved therapeutic efficacy can be achieved through dual incretin receptor agonism, further optimisation is still required.

Biased agonism presents a second alternative to optimise incretin-based therapeutics. Here, ligands stabilise the receptor conformation in such a way that one or more signalling pathways are selectively activated, whilst others are reduced or ablated altogether. The consequent signalling profile, which is “biased” toward certain pathways over others, allows pathways associated with the therapeutic effect of receptor activation to be accentuated over those which elicit side effects, resulting in a more efficacious and tolerable pharmacotherapy. Human studies have confirmed the value and potential of biased agonists compared to traditionally used “balanced” agonists such as morphine at the μ-opioid receptor[490,492,493].

It has been shown in recent years that G protein-biased GLP-1R agonists display greater anti-hyperglycaemic properties and tolerability than unbiased equivalents, with matched[276,502] or even greater[504] weight loss and food intake. These data confirm that bias can be leveraged with incretin treatments to improve anti-diabetic efficacy. However, no work has been reported describing the effects of biased dual GLP-1R/GCGR agonism. Therefore, in addition to the improved metabolic outcomes of dual GLP-1R/GCGR agonists, a biased dual agonist could theoretically yield greater weight loss and/or superior glycaemic control, due to an increased tolerability thus providing a novel therapeutic strategy to treat the obese T2DM epidemic.

In this project, an example of a dual agonist displaying low efficacy for β-arrestin-2 recruitment, with full cAMP signalling, was identified alongside a comparator with full β-arrestin signalling. This compound was validated for glucoregulatory and weight loss effects to confirm whether favouring signalling through G protein-related pathways over β-arrestin recruitment improves the therapeutic effects of the dual agonist.

6.1 Identification of lead compounds

Biased candidate ligands were initially selected by identifying dual agonists with corresponding peptides containing single amino acid substitutions. This strategy was predicated on the reasoning that single amino acid substitutions were unlikely to greatly affect physico-chemical properties or proteolytic stability and resultant pharmacokinetics, which was confirmed by radioimmunoassay and HPLC (Figure 4.2F & G). This approach has been used previously to identify biased Ex4-based GLP-1R agonists[276]. Biased signalling was identified using a simple medium-throughput screen, whereby single, high-dose β-arrestin recruitment was examined (Figure 3.2). This data was then cross-referenced to historical cAMP signalling data for the selected peptides, and XY linear regression analysis used to identify hit compounds with differential profiles for cAMP production or β-arrestin recruitment (Figure 3.2B). Whilst this strategy is limited in terms of providing a formal pharmacological comparison, as it compares two separate pharmacological metrics which are not inherently related (potency for cAMP *versus* presumed efficacy for β-arrestin), this pragmatic and resource-effective approach provided hit compounds for more formal validation. The

substitution of AIB at position 2 of the molecule (AIB2Gln3) is a commonly used amino acid substitution to prevent DPP-IV degradation[298]. However, when substituted into native OXM, it resulted in reduced β-arrestin recruitment at the GCGR compared to glucagon, whilst maintaining robust cAMP potency yet acted as a full and potent agonist for both pathways at the GLP-1R (Figure 3.2C). When the Gln3 was substituted for histidine (His3), the profiles of potency and efficacy for cAMP and β-arrestin was returned to that of the endogenous ligand. This mimics the *in vitro* profile of Ex-phe1 *versus* Ex4 at the GLP-1R, which provided the theoretical basis behind the work presented here[276].

This suggests that the AIB2Gln3 amino acid sequence produces an agonist biased toward cAMP production over β-arrestin recruitment compared to AIB2His3. According to the canonical role of β-arrestins, this should theoretically produce a peptide with reduced GCGR desensitisation and internalisation[276], prolonging GCGR signalling. Previous studies have highlighted the metabolic benefits of GCGR signalling including improvements to weight loss and insulin secretion and insulin sensitivity[227,228,382,394]. Hence, potentially prolonged GCGR signalling produced by AIB2Gln3 peptides might result in a novel and superior anti-diabetic and anti-obesity therapy. Therefore, two lead compounds containing the AIB2Gln3 and AIB2His3 amino acid switch were produced, called SRB103Gln3 and SRB103His3. Both of these SRB103 compounds displayed similar signalling patterns as observed in the endogenous OXM-based peptides, but SRB103Gln3 was equipotent for cAMP production at the GCGR. This meant that the most prominent signalling difference observed in the Cisbio cAMP assay and PathHunter β-arrestin recruitment assay was reduced β-arrestin recruitment at the GCGR (Figure 3.3A). In Huh7 hepatoma cells overexpressing GCGR, 16-hour stimulation with SRB103Gln3 resulted in much greater cAMP production compared to SRB103His3, suggesting that reduced β-arrestin recruitment with SRB103Gln3 at the GCGR leads to reduced receptor desensitisation, possibly reduced internalisation, and ultimately a prolongation of receptor signalling (Figure 3.5A). However, in HEK293T cells transiently transfected with either GCGR or GLP-1R, there was not an observable difference in prolonged signalling, suggesting that either the prolonged signalling effect *in vitro* is a cell-specific phenomenon, or that the cell line differences are linked to surface receptor expression.

Concentration-response data for both of these compounds were analysed for quantitative bias using current methods, including a modified version of the operational model of agonism and the relative activity scale (Figure 3.3B & C). However, neither of the methods used confirmed SRB103Gln3 as being significantly biased toward cAMP signalling over β-arrestin recruitment at the GCGR compared to SRB103His3. The operational model has limitations when quantifying very partial agonists[479] and with very similar potencies at the receptors between the two compounds, the method does not sufficiently discriminate between a full agonist and partial agonist with similar potencies. The relative activity scale similarly failed to confirm evidence of statistically significant signal bias. In spite of the apparent lack of formally confirmed signal bias, the Gln3 compounds reproducibly showed a characteristic pattern of intracellular signalling characterised by reduced β-arrestin recruitment efficacy at the GCGR whilst maintaining full efficacy for the amplified cAMP pathway.

The cAMP *versus* β-arrestin recruitment assay in PathHunter cells is inherently limited by non-physiological, irreversible recruitment of β-arrestin to the receptor, along with reduced discrimination between G protein events due to amplification in the G_s-AC-cAMP signalling pathway. Therefore, dynamic recruitment of a mini $G\alpha_s$ or β-arrestin-2 construct to the GLP-1R or GCGR was analysed using the NanoBiT assay (Figure 3.4). The results diverge from those of the Cisbio and PathHunter assays, in that it showed SRB103Gln3 displayed equal potency and efficacy for $G\alpha_s$ recruitment but reduced β-arrestin recruitment at the GLP-1R (i.e. biased toward $G\alpha_s$ recruitment) compared to SRB103His3, but lower in efficacy for both pathways at the GCGR (i.e. a partial agonist). These system-specific response patterns pose issues for the correct assignment of signal preference for each ligand. The increase in prolonged GCGR-mediated cAMP signalling in Huh7 hepatoma cells with SRB103Gln3 signals could be explained by the pharmacological characteristics suggested by either the PathHunter or NanoBiT data, with reduced β-arrestin recruitment at the GCGR a reasonable explanation for the apparently reduced desensitisation response[276]. However, the acute and chronic *in vivo* studies show that SRB103Gln3 treatment produces longer lasting glycaemic control, mimicking that of a biased GLP-1R agonist (discussed in chapters 4 and 5). Moreover, the absence of successful antagonist studies, due to a lack of *in vivo* efficacy of the antagonist ligands designed to assess this (Figure 4.4), makes delineation more difficult. Preliminary tests using the rat β cell line INS1 832/3

which had either the GLP-1R or GIPR knocked out using CRISPR-Cas9 suggest that removal of GLP-1R signalling has a more significant impact on SRB103Gln3 mediated insulin secretion compared to SRB103His3 (data not shown) whilst the GIPR appears more important to SRB103His3-mediated insulin release. This requires much closer investigation as it does further implicate the GLP-1R as being central to SRB103Gln3-mediated insulinotropic improvements seen in this work, as well as suggesting mild tri-agonist abilities of the SRB103 peptides.

As is suggested by the Huh7 data and evidenced by other examples of biased agonists which diminished β-arrestin recruitment, mitigating β-arrestin recruitment at either the GLP-1R or GCGR may result in reduced β-arrestin-mediated internalisation[276,826]. This would allow a greater proportion of receptor to remain on the cell surface at steady state after prolonged stimulation, facilitating ongoing receptor signalling. In the example of GLP-1R, it is known that reduced receptor internalisation increases chronic anti-hyperglycaemic capabilities of the biased compound[276,502,504]. In the case of the GCGR, knockout of β-arrestin-2 results in prolonged receptor signalling by reducing receptor internalisation[283]. As GCGR stimulation is also associated to improved metabolic outcomes, SRB103Gln3 may impart beneficial therapeutic effects by reducing β-arrestin-mediated receptor internalisation at either the GLP-1R or GCGR. To investigate this, fluorescent-tagged GLP-1R or GCGR were stimulated with either SRB103 peptide to visualise the compartmentalisation of the receptors using wide-field microscopy. Interestingly, contrary to expectations, SRB103Gln3 did not cause a visible reduction in internalisation at either receptor (Figure 3.6). This could result from a number of factors. Firstly, whilst there may indeed be a subtle difference in internalisation, a microscopy assay based on subjective review of distribution of labelled intracellular receptor after agonist stimulation may be insufficiently sensitive to identify this difference. Secondly, the extent of reduction in β-arrestin recruitment produced by SRB103Gln3 stimulation is less than for other G-protein biased GLP-1R agonists showing dramatically reduced agonist-mediated receptor internalisation[276,502]. Cumulatively, the *in vitro* assays show that SRB103Gln3 is still able to recruit β-arrestins at worst 50% as efficiently as SRB103His3 (Figure 3.3A), or as much as 78% (Figure 3.4C & D), meaning that there may still be enough β-arrestin recruitment to initiate receptor internalisation. Thirdly, the efficiency of receptor transfection in this assay may be poor and result in a greater ratio of β-arrestin-to-

receptor to that found in the Huh7 cells, resulting in a greater prevalence of β-arrestin binding to the receptor and producing internalisation. This could mask the true events that occur *in vitro*. Other differences related to expression of trafficking biomolecules in different cell types are also a possible contributory factor. Finally, the mechanism by which the reduced β-arrestin recruitment pattern of SRB103Gln3 allows prolonged GCGR signalling may be separate from internalisation, reflecting instead reduced steric hinderance of G protein interactions at the plasma membrane. This requires greater delineation, and work performed in this laboratory using fluorophore cleavage can be used to further investigate receptor recycling rates[276,827,828].

In summary, a dual agonist (SRB103Gln3) was discovered and validated using a wide range of *in vitro* techniques which suggested preferential G protein-directed signalling at the GLP-1R according to one experiment, or at the GCGR according to the other. Separate methods of bias quantification failed to determine statistical differences in bias between SRB103Gln3 and SRB103His3. However, clear prolongation of signalling shown at the GCGR in Huh7 cells suggested the reduced β-arrestin recruitment pattern was functionally important. No gross differences in GLP-1R or GCGR internalisation were observed between SRB103Gln3 *versus* SRB103His3, but other trafficking phenomena, such as recycling or post-endocytic sorting to difference subcellular compartments, were not investigated.

6.2 Dual GLP-1R/GCGR agonist with reduced β-arrestin efficacy improves anti-hyperglycaemic response

Having identified SRB103Gln3 as a ligand displaying selective signalling toward the G protein/cAMP pathway with diminished β-arrestin recruitment, with SRB103His3 as the unbiased comparator, extensive *in vivo* validation was performed. In keeping with G protein-biased GLP-1R agonists[276], SRB103Gln3 and SRB103His3 displayed similar acute anti-hyperglycaemic abilities (Figure 4.2A & B). However, four- and eight-hours post peptide administration, SRB103Gln3 displayed superior anti-hyperglycaemic abilities. This phenotype was replicated using four further Gln3 and His3 peptide pairs (some were not numerically significant, however) further validating the usefulness of G protein bias in dual agonists toward improved treatments for

diabetes. This was not related to pharmacokinetics (Figure 4.2F), but appeared to result from an increase in sustained insulinotropic ability of SRB103Gln3 compared to SRB103His3 (Figure 4.2C). This is in keeping with other studies investigating G protein-bias at the GLP-1R$_{276,504}$, suggesting that SRB103Gln3 acts in this way also. No observable changes in insulin tolerance was observed (Figure 4.2D & E), suggesting the acute anti-hyperglycaemic effects of SRB103Gln3 relate primarily to increased insulin secretion. Whether this is a primary attribute of biased signalling at one of the receptors or secondary to an increase in glucagon-mediated glycaemia through enhanced GCGR signalling is not clear. Interestingly, in *Gcgr*$^{-/-}$ and *Gcgr*$^{hep-/-}$ mice, the acute four-hour anti-hyperglycaemic ability of SRB103His3 and vehicle matched that of SRB103Gln3 (Figure 4.8), suggesting that reducing GCGR signalling improves anti-hyperglycaemic responses as would be attributed to SRB103Gln3 if it acted as a partial agonist at the GCGR (as suggested in the NanoBiT assay). However, the physiology of these transgenic mouse lines features various adaptive mechanisms which could confound valid interpretation of the results. Acute blockade of either receptor using antagonists was attempted, but their resulting *in vivo* efficacies were insufficient to adequately ablate the activity of GLP-1R or GCGR mono-agonists, even at relatively low pharmacological doses (Figure 4.4). Monoclonal blocking antibodies are a further option which could be tried, but they also tend to show only partial effects against potent pharmacological agonists.

In studies of chronic administration to mice, SRB103Gln3 continued a trend toward greater anti-hyperglycaemic control compared to both SRB103His3 and GLP-1R mono-agonist liraglutide. Differences between SRB103Gln3 and SRB103His3 were not statistically significant, however this is likely related to higher dosing than that used in the acute studies. At a high dose (50nmol/kg) both SRB103 peptides significantly reduced glucose-induced hyperglycaemia (Figure 5.3A), which coincided with a large reduction in body weight and food intake. At a lower dose (20nmol/kg), there is a much milder reduction in body weight by the end of the study, however SRB13Gln3 still produced the same level of anti-hyperglycaemia, whereas the efficacy of SRB103His3 reduced (Figure 5.3B). This indicates that SRB103Gln3 elicits its anti-hyperglycaemic abilities separate from that of body weight change and food intake. SRB103Gln could, therefore, be useful in clinical situations where weight loss is of secondary importance to glycaemic control, such as overweight (i.e. not obese) patients with T2DM. Further

investigation into dose accumulation, insulin secretion and translation into transgenic mice to further understand the mechanism behind the observed phenotype of SRB103Gln3 mice is required.

This work is limited by the lack of *in vitro* work performed in mouse cell lines, therefore the difference in receptor potencies between SRB103 peptides at both mouse receptors is unknown. Due to differences in sequence homology between the human and mouse GLP-1R and GCGR, it is possible that the phenotypes witnessed here are linked to SRB103Gln3 being more potent at the mouse GLP-1R than SRB103His3. That would produce the same hyperglycaemic phenotype, but it does not explain the other *in vivo* phenotypes witnessed. Further investigation of murine receptor signalling and extensive use of a range of transgenic mouse lines are required to fully corroborate the anti-hyperglycaemic data produced in this work. Indeed, it has been reported that biased GLP-1R agonists display improved chronic anti-hyperglycaemic abilities in mice[276,502,504], and the data from this work supports this hypothesis. In addition to superior metabolic effects compared to an unbiased dual agonist comparator, SRB103Gln3 showed much greater chronic anti-hyperglycaemic abilities than liraglutide, a GLP-1R agonist marketed for diabetes and obesity, further highlighting the exciting potential that SRB103Gln3 displays for tackling obese-related T2DM.

GCGR activation leads to increased glycaemia, and patients with T2DM typically have hyperglucagonaemia[356]. This has led to the theory the glucagon could be the key hormonal driver of diabetes, and indeed, *Gcgr*$^{-/-}$ mice are resistant to the onset of diabetes[829,830] resulting in the development of GCGR antagonists[733,734]. However, the repeated lack of success of GCGR antagonists in clinical trials demonstrates that insulin signalling is more likely the key driver to the progressive deterioration of glucose control witnessed in T2DM. Additionally, prolonged GCGR activation has been shown to increase insulin secretion and sensitivity and may improve the insulin response to hyperglycaemia[394]. These data are supported by my work, which demonstrated the improvements in chronic anti-hyperglycaemia in DIO mice treated with SRB103 compound compared to the GLP-1R mono-agonist liraglutide, which was distinct from their superior abilities to improve weight loss as well.

In summary, this work links the molecular bias of SRB103Gln3 to an improvement in anti-hyperglycaemic response. This effect is dose related and is lost at higher doses which produce profound weight loss.

6.3 Dual GLP-1R/GCGR agonist with reduced β-arrestin efficacy does not further decrease body weight or food intake

Another key *in vivo* parameter investigated was the ability of the SRB103 peptides to induce body weight loss and reduce food intake. Many pharmacotherapies marketed for the treatment of T2DM can result in body weight increases, which is not ideal in T2Dm as the majority of patients are overweight[8]. Not only does co-agonism of GLP-1R and GCGR reduce resting glycaemia in humans, it also results in robust weight loss, which further improves insulin sensitivity[227,228,430,431,438,439,441,443,444].

In this work, chronic studies were performed to investigate the chronic glucoregulatory effects of SRB103 peptides and liraglutide. Therefore, to mitigate changes in body weight as a confounder for IPGTT performed at the end of the study, these were performed when all peptide-treated groups were weight matched. At 50nmol/kg, by the end of the monitoring period (Day 18), SRB103His3- and SRB103Gln3-treated mice had lost 8.7 and 8.9g respectively (Figure 5.1A), suggesting SRB103Gln3 had no direct effect on body weight change compared to SRB103His3, a phenotype that biased GLP-1R agonists have shown *versus* unbiased comparators[276,502,520]. Meanwhile, liraglutide-treated mice showed a 7.0g loss and the weight loss was plateauing, whilst SRB103-treated mice were continuing to lose weight. This emphasises that the additional GCGR-mediated effect on energy expenditure with dual GLP-1R/GCGR agonists can result in a superior weight loss compared to GLP-1R agonists alone. In the 20nmol/kg study, all peptide-treated groups had lost less weight by day 15, when the study ended for the IPGTT (6.4g, 4.9g & 5.7g for liraglutide, SRB103His3 and SRB103Gln3 respectively) (Figure 5.1B). However, the trajectory of weight loss was as in the 50nmol/kg where liraglutide-mediated weight loss had plateaued whereas SRB103Gln3 and -His3 treatment was still producing weight loss. It can therefore be anticipated that, if these studies were further prolonged, the weight loss resulting from the SRB103 peptides could be even greater than with liraglutide,

although it appears likely that a similar weight loss would be achieved with SRB103His3 and SRB103Gln3.

In conjunction with body weight change, food intake was also measured. In both chronic mouse studies, SRB103Gln3 and -His3-treated mice ate more than liraglutide treated mice (Figure 5.2) and although the difference was small and, in most cases, insignificant, it was replicable. There was no difference in food intake between the SRB103 peptides chronically, which replicates other chronic studies using biased GLP-1R agonists[276,502,520]. This phenotype is interesting, suggesting that G protein-bias at the GLP-1R does not prolong all GLP-1R-mediated phenotypes, and is a definite case for further investigation for future therapeutic optimisation. It also suggests that the reduced efficacy for β-arrestin recruitment produced by SRB103Gln3 does not affect tolerability of the compound. This is exciting as it suggests for a similar amount of tolerability (i.e. food intake) SRB103Gln3 can produce a greater anti-hyperglycaemic effect. The trends established in the chronic study mimic those seen in the acute 8-hour studies in DIO mice, which showed a consistent reduction in food intake with SRB103Gln3 and SRB103His3 (Figure 4.10B). In contrast, acute feeding studies in lean mice suggest that SRB103Gln3 reduces food intake more than SRB103His3 treatment (Figure 4.10A). This difference in acute food intake between the two mouse cohorts is perhaps related to the dosage of the compounds, which were calculated using total body weight. Therefore, DIO mice received a greater total amount of peptide, which as discussed in section 4.1.3, could result in a greater circulating drug concentration and thus result in SRB103His3 becoming more anorexigenic. The precise mechanisms linking incretin dose, receptor potencies, bias and food intake are poorly understood, and certainly require more investigation using antagonists and transgenic mouse lines.

Interestingly, there was a marked difference between results observed in mice and rats. In rats, at 6- and 12nmol/kg, SRB103Gln3 treatment resulted in a 34% and 45% reduction in food intake over three days compared to SRB103His3 (Figure 5.5B & C). There were statistically similar, large reductions in body weight of 34g and 35g for SRB103Gln3 and SRB103His3 at 6nmol/kg and 45g and 38g for SRB103Gln3 and SRB103His3 at 12nmol/kg. The weight loss seen with both SRB103 peptides was greater than that of liraglutide, further highlighting the exciting potential of dual incretin

receptor agonists as a treatment for weight loss. This also suggests that, in rats, SRB103Gln3 reduces weight primarily through reduction of food intake, whereas SRB103His3 results in greater energy expenditure. Evidence from our laboratory suggest that GLP-1R-favouring agonists cause greater food intake reduction and increased diuresis, whereas GCGR favouring compounds eat more but lose more weight through increased energy expenditure. Those findings are repeated here, and the extent to which G protein GLP-1R bias is affecting food intake with SRB103Gln3 is worth further consideration, as are the effects of differential GCGR signalling on weight loss, food intake and glucose control in rats.

One possible explanation for the different effects of the SRB103 peptides in mice and rats may relate to “tissue bias”. Like system bias, this reflects the ability of compounds to uniquely activate signalling pathways in different tissues (or the same tissue of different species), corresponding to tissue-specific microenvironment which includes different expression levels of signal transducers. It is known that different species can display unique responses to the same ligand at the same receptor[813,814]. In this instance, SRB103Gln3 could act in mice to initiate energy expenditure (perhaps through BAT browning) whilst in rats, the specific intracellular environment in their BAT does not couple sufficiently to and allow for SRB103Gln3-mediated energy expenditure. Additionally, the sensitivity of different physiological actions of GCGR signalling may explain why SRB103Gln3 does not instigate energy expenditure in rats whereas SRB103His3 does. Here, some physiological responses to GCGR signalling, such as gluconeogenesis, may be very sensitive to GCGR signalling whilst others, such as GCGR-mediated energy expenditure may be less sensitive. As SRB103Gln3 is a low efficacy GCGR ligand, it may be that SRB103Gln3 is not efficacious enough to stimulate GCGR-mediated energy expenditure in rats whereas SRB103His3, a full agonist at the GCGR. Therefore, even if the desensitisation produced at the GCGR between the two SRB103 peptides is different, SRB103Gln3 still would not be able to elicit GCGR-mediated energy expenditure as its intrinsic efficacy is not large enough. Whilst these theories are interesting, further delineation is required by evaluating the pharmacological signalling profile of SRB103Gln3 and SRB103His3 at both the rat and mouse GLP-1R and GCGR orthologues, and then aligning GCGR physiology in each species with the given pharmacological profile.

In summary, there was no difference in body weight reductions observed between the SRB103 peptides in either species. In mice, both SRB103 peptides produced a lesser reduction in food intake compared to liraglutide for the same weight loss, suggesting increased energy expenditure. In rats, the prolonged GLP-1R signalling and partial GCGR agonism causes great reductions in food intake for SRB103Gln3-treated rats, whilst reducing the G protein GLP-1R bias and increasing the GCGR signalling quantity with SRB103His3 causes similar weight loss with much greater food intake, indicative of increased energy expenditure.

6.4 Summary, Scope and Future Implications

The data presented in this work emphasises the exciting potential that developing biased GPCR agonists could bring to future disease targets.

Displaying superior anti-hyperglycaemic properties compared to the unbiased equivalent peptide SRB103His3, and greater chronic weight loss than the GLP-1R mono-agonist liraglutide, SRB103Gln3 has demonstrated potential as a novel therapeutic for the treatment of T2DM in obese patients. However, with no change in acute food intake compared to SRB103His3 in mice and in rats, it appears that this is unlikely to be accompanies by increased nausea, suggesting tolerability is relatively improved[326].

Obesity is closely linked to other metabolic diseases including non-alcoholic fatty liver disease (NAFLD), in which excessive lipid build up can result in liver cirrhosis[831]. Whilst late stage cirrhosis is currently untreatable, attempts to inhibit the early stage of fatty liver is of critical importance. GLP-1R agonists, alongside their well-described effects on weight loss and diabetes control, have displayed exciting potential to improve hepatic lipid stores after chronic treatment, and G protein-biased GLP-1R have shown an even greater therapeutic ability than unbiased comparators[276,502,504]. As SRB103Gln3 displays G-protein bias at the GLP-1R compared to SRB103His3, it could be hypothesised that SRB103Gln3 would be an exciting therapeutic candidate to treat early stage fatty liver. In addition to its biased signalling, SRB103Gln3 also signals through the GCGR, which has itself been associated with improving fatty liver.

Dual GLP-1R/GCGR agonists have been described as effective agents to reverse fatty liver development[436,801,802], and the discovery of this G protein-biased dual GLP-1R/GCGR agonist may provide an even more powerful tool to tackle NAFLD, and is worth exploring in the future. GLP-1R agonists have also been implicated in other disease areas, such as cardiovascular disease[832] and CNS disease[833]. As metabolism is such a key physiological event for all cell types, tissues and organs, the direct or indirect effects of dual agonists and biased signalling is exciting and is worthy of further study.

An unexplored problem associated with dual GLP-1R/GCGR agonist treatment is the observed increase in lean mass loss, attributable to GCGR activation. In diabetic patients, lean mass is a critical tissue to dispose of the high circulating levels of glucose[531,532]. Glucagon stimulates hepatic gluconeogenesis by increasing the flux of glucogenic amino acids into the liver which results in hypoaminoacidaemia and ultimately protein catabolism[350]. This was witnessed in the chronic studies performed in this work, where mice displayed greater lean mass loss after SRB103 peptide treatment compared to GLP-1R agonist liraglutide (Figure 5.4). Interestingly, the reduced efficacy at the GCGR produced by SRB103Gln3 had no effect on lean mass loss. In cases of increased circulating glucagon levels, such as glucagonoma patients, the risk of excessive muscle wasting is greatly increased[350,351,357]. In this study, we observe a greater lean mass loss produced by low dose SRB103 agonist treatment compared to a similar dose of liraglutide, highlighting the potential risks of GCGR stimulation in diabetic patients, where glucose sequestration into lean mass is already compromised. Further increasing lean mass loss in diabetic patients is therefore a possible limiting factor against their widespread use. Whether alterations to the signalling profile of the dual GLP-1R/GCGR agonist reduce the tendency for lean mass loss, such as making the agonist more GLP-1R-favouring or intelligently designed biased agonism, is further important work that requires investigation.

As described in the chapters beforehand, this work builds on earlier work implicating G protein-bias at the GLP-1R as metabolically advantageous in the fight against T2DM and obesity. The principle driving the search for biased peptides rests on the canonical role of β-arrestins in terminating G protein signalling, meaning that agonists which minimise the former pathway may allow increased duration of signalling in metabolic

tissues. Another method to achieve this is to increase the dose or increase the potency of the ligand to the receptors. However, these two methods do not circumvent the intrinsic problem with these OXM- or GLP-1R-based ligands: they induce nausea at doses which limit their therapeutic usage. Creating more potent peptides or increasing the dose will result in the same problems that conventional ligands possess. Therefore, the method of reducing the amplitude of signalling of pathways which create side effect profiles is a method which can actually improve therapeutic output, as demonstrated by MOR agonist TRV130 in humans[490-493].

The process of taking a drug concept from bench top to clinic is time- and resource-intensive. If SRB103Gln3 were to undergo further preclinical and ultimately clinical development, a considerable amount of further investigation will be required. Firstly, alterations to amino acid sequence and addition of macromolecules such as cholesterol, PEG, or a fatty acid side chain, is required to extend the circulating half-life of SRB103Gln3, whilst not compromising receptor potency (see section 5.1.1). In accordance with these changes to the peptide structure, assessment of other "druggable" properties of SRB103Gln3 will also need to be performed. The assessment of the solubility and stability of the peptide in therapeutic diluent can be done using HPLC or mass spectroscopy analysis, as performed in our laboratory. After a potent, selective, PK-enhanced peptide is found, testing of SRB103Gln3 pharmacokinetics, toxicology, tolerability, efficacy and dosage prediction is performed in a rodent and non-rodent species. Once the peptide is shown to be a "druggable" peptide both *in vitro* and *in vivo*, data is collated into an Investigator's Brochure for the MHRA application for clinical trials. Even if the MHRA application is granted, the rate of drug failure in clinical trials is great[834], therefore the likelihood of SRB103Gln3 progressing to clinic is low.

In conclusion, this project has identified the first reported examples of G protein-favouring dual GLP-1R/GCGR agonists, and the biased agonist displays greater acute anti-hyperglycaemic properties due to an increase in insulin secretion *in vivo*. Whilst there was no significant difference in weight loss or tolerability chronically, the G protein-biased compound showed improved anti-hyperglycaemic effects *versus* an unbiased comparator compound. This work, therefore, validates G protein/cAMP bias

for OXM-based dual agonist therapies as a novel method of improving therapeutic efficacy, and merits further investigation.

7 Appendix

Peptide sequences:

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42
GLP-1(7-37)	H	A	E	G	T	F	T	S	D	V	S	S	Y	L	E	G	Q	A	A	K	E	F	I	A	W	L	V	K	G	R												
GCG	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	S	R	R	A	Q	D	F	V	Q	W	L	M	N	T	K	R	N	R	N	I	A					
Oxyntomodulin	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	S	R	R	A	Q	D	F	V	Q	W	L	M	N	T	K	R	N	R	N	I	A					
GIP	Y	A	E	G	T	F	I	S	D	Y	S	I	A	M	D	K	I	H	Q	Q	D	F	V	N	W	L	L	A	Q	K	G	K	K	N	D	W	K	H	N	I	Q
OXM AIB2	H	X	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	S	R	R	A	Q	D	F	V	Q	W	L	M	N	T	K	R	N	R	N	N	I	A					
OXM His3	H	S	H	G	T	F	T	S	D	Y	S	K	Y	L	D	S	R	R	A	Q	D	F	V	Q	W	L	M	N	T	K	R	N	R	N	N	I	A					
OXM AIB2His3	H	X	H	G	T	F	T	S	D	Y	S	K	Y	L	D	S	R	R	A	Q	D	F	V	Q	W	L	M	N	T	K	R	N	R	N	N	I	A					
SRB103His3	H	X	H	G	T	F	T	S	D	Y	S	K	Y	L	D	A	K	R	A	Q	E	F	I	E	W	L	L	A	G	H	H	H	H	H	P	S	W				
SRB103Gln3	H	X	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	A	K	R	A	Q	E	F	I	E	W	L	L	A	G	H	H	H	H	H	P	S	W				
Acyl-Ex9									D	L	S	K	Q	M	E	E	E	A	V	R	L	F	I	E	W	L	K	N	G	G	P	S	S	G	A	P	P	S	G	yLys	Hexadecanedioic (C16) acid
Acyl-DHG		S	Q	G	T	F	T	S	E	Y	S	K	Y	L	D	S	R	R	A	Q	D	F	V	Q	W	L	M	N	T	yLys	G	S	G	yGlu	Eicosanedioic (C20) acid							
His3Tyr1	Y	X	H	G	T	F	T	S	D	Y	S	K	Y	L	D	A	K	R	A	Q	E	F	I	E	W	L	L	A	G	H	H	H	H	H	P	S	W				
His3Ser18	H	X	H	G	T	F	T	S	D	Y	S	K	Y	L	D	A	K	S	A	Q	E	F	I	E	W	L	L	A	G	H	H	H	H	H	P	S	W				
Gln3Tyr1	Y	X	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	A	K	R	A	Q	E	F	I	E	W	L	L	A	G	H	H	H	H	H	P	S	W				
Gln3Ser18	H	X	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	A	K	S	A	Q	E	F	I	E	W	L	L	A	G	H	H	H	H	H	P	S	W				

8 Bibliography

1 Rankovic Z., Brust T. F., Bohn L. M. Biased Agonism: An Emerging Paradigm in Gpcr Drug Discovery. Bioorg Med Chem Lett. 2016;26(2):241-50.

2 Müller T. D., Finan B., Clemmensen C.*, et al.* The New Biology and Pharmacology of Glucagon. Physiol Rev. 2017;97(2):721-66.

3 Kenakin T., Christopoulos A. Signalling Bias in New Drug Discovery: Detection, Quantification and Therapeutic Impact. Nat Rev Drug Discov. 2013;12(3):205-16.

4 Brownlee M. A Radical Explanation for Glucose-Induced Beta Cell Dysfunction. J Clin Invest. 2003;112(12):1788-90.

5 Federation I. D. Idf Diabetes Atlas, 9 Edn. Brussels, Belgium2019 [Available from: https://www.diabetesatlas.org.

6 Murphy S. L., Xu J., Kochanek K. D.*, et al.* Deaths: Final Data for 2015. Natl Vital Stat Rep. 2017;66(6):1-75.

7 Saeedi P., Petersohn I., Salpea P.*, et al.* Global and Regional Diabetes Prevalence Estimates for 2019 and Projections for 2030 and 2045: Results from the International Diabetes Federation Diabetes Atlas, 9(Th) Edition. Diabetes Res Clin Pract. 2019;157:107843.

8 World Health Organisation. Diabetes 2020 [Available from: https://www.who.int/news-room/fact-sheets/detail/diabetes.

9 Bommer C., Sagalova V., Heesemann E.*, et al.* Global Economic Burden of Diabetes in Adults: Projections from 2015 to 2030. Diabetes Care. 2018;41(5):963-70.

10 Arrojo e Drigo R., Ali Y., Diez J.*, et al.* New Insights into the Architecture of the Islet of Langerhans: A Focused Cross-Species Assessment. Diabetologia. 2015;58(10):2218-28.

11 Röder P. V., Wu B., Liu Y.*, et al.* Pancreatic Regulation of Glucose Homeostasis. Exp Mol Med. 2016;48(3):e219.

12 Svendsen B., Larsen O., Gabe M. B. N.*, et al.* Insulin Secretion Depends on Intra-Islet Glucagon Signaling. Cell Rep. 2018;25(5):1127-34.e2.

13 Kawamori D., Kurpad A. J., Hu J.*, et al.* Insulin Signaling in Alpha Cells Modulates Glucagon Secretion in Vivo. Cell Metab. 2009;9(4):350-61.

14 Campfield L. A., Smith F. J. Neural Control of Insulin Secretion: Interaction of Norepinephrine and Acetylcholine. Am J Physiol. 1983;244(5):R629-34.

15 Newsholme P., Krause M. Nutritional Regulation of Insulin Secretion: Implications for Diabetes. Clin Biochem Rev. 2012;33(2):35-47.

16 Brown J. C., Otte S. C. Gastrointestinal Hormones and the Control of Insulin Secretion. Diabetes. 1978;27(7):782-7.

17 Nauck M. A., Homberger E., Siegel E. G.*, et al.* Incretin Effects of Increasing Glucose Loads in Man Calculated from Venous Insulin and C-Peptide Responses. J Clin Endocrinol Metab. 1986;63(2):492-8.

18 Holst J. J. From the Incretin Concept and the Discovery of Glp-1 to Today's Diabetes Therapy. Front Endocrinol (Lausanne). 2019;10:260.

19 Baggio L. L., Drucker D. J. Biology of Incretins: Glp-1 and Gip. Gastroenterology. 2007;132(6):2131-57.

20 Vilsbøll T., Krarup T., Madsbad S.*, et al.* Both Glp-1 and Gip Are Insulinotropic at Basal and Postprandial Glucose Levels and Contribute Nearly Equally to the Incretin Effect of a Meal in Healthy Subjects. Regul Pept. 2003;114(2-3):115-21.

21 Nauck M. A., Heimesaat M. M., Orskov C.*, et al.* Preserved Incretin Activity of Glucagon-Like Peptide 1 [7-36 Amide] but Not of Synthetic Human Gastric Inhibitory Polypeptide in Patients with Type-2 Diabetes Mellitus. J Clin Invest. 1993;91(1):301-7.

22 Gasbjerg L. S., Christensen M. B., Hartmann B.*, et al.* Gip(3-30)Nh(2) Is an Efficacious Gip Receptor Antagonist in Humans: A Randomised, Double-Blinded, Placebo-Controlled, Crossover Study. Diabetologia. 2018;61(2):413-23.

23 Gasbjerg L. S., Helsted M. M., Hartmann B.*, et al.* Separate and Combined Glucometabolic Effects of Endogenous Glucose-Dependent Insulinotropic Polypeptide and Glucagon-Like Peptide 1 in Healthy Individuals. Diabetes. 2019;68(5):906-17.

24 Carr R. D., Larsen M. O., Winzell M. S.*, et al.* Incretin and Islet Hormonal Responses to Fat and Protein Ingestion in Healthy Men. Am J Physiol Endocrinol Metab. 2008;295(4):E779-84.

25 Seino Y., Fukushima M., Yabe D. Gip and Glp-1, the Two Incretin Hormones: Similarities and Differences. J Diabetes Investig. 2010;1(1-2):8-23.

26 Suzuki K., Harada N., Yamane S.*, et al.* Transcriptional Regulatory Factor X6 (Rfx6) Increases Gastric Inhibitory Polypeptide (Gip) Expression in Enteroendocrine K-Cells and Is Involved in Gip Hypersecretion in High Fat Diet-Induced Obesity. J Biol Chem. 2013;288(3):1929-38.

27 Yamane S., Harada N., Hamasaki A.*, et al.* Effects of Glucose and Meal Ingestion on Incretin Secretion in Japanese Subjects with Normal Glucose Tolerance. J Diabetes Investig. 2012;3(1):80-5.

28 Kozawa J., Okita K., Iwahashi H.*, et al.* Early Postprandial Glucagon Surge Affects Postprandial Glucose Levels in Obese and Non-Obese Patients with Type 2 Diabetes. Endocr J. 2013;60(6):813-8.

29 Tang C., Naassan A. E., Chamson-Reig A.*, et al.* Susceptibility to Fatty Acid-Induced B-Cell Dysfunction Is Enhanced in Prediabetic Diabetes-Prone Biobreeding Rats: A Potential Link between B-Cell Lipotoxicity and Islet Inflammation. Endocrinology. 2013;154(1):89-101.

30 Donath M. Y., Dalmas É., Sauter N. S.*, et al.* Inflammation in Obesity and Diabetes: Islet Dysfunction and Therapeutic Opportunity. Cell Metab. 2013;17(6):860-72.

31 Rösen P., Nawroth P. P., King G.*, et al.* The Role of Oxidative Stress in the Onset and Progression of Diabetes and Its Complications: A Summary of a Congress Series Sponsored by Unesco-Mcbn, the American Diabetes Association and the German Diabetes Society. Diabetes Metab Res Rev. 2001;17(3):189-212.

32 Cen J., Sargsyan E., Bergsten P. Fatty Acids Stimulate Insulin Secretion from Human Pancreatic Islets at Fasting Glucose Concentrations Via Mitochondria-Dependent and -Independent Mechanisms. Nutr Metab (Lond). 2016;13(1):59.

33 Straub L. G., Efthymiou V., Grandl G.*, et al.* Antioxidants Protect against Diabetes by Improving Glucose Homeostasis in Mouse Models of Inducible Insulin Resistance and Obesity. Diabetologia. 2019;62(11):2094-105.

34 Ahrén B. Reducing Plasma Free Fatty Acids by Acipimox Improves Glucose Tolerance in High-Fat Fed Mice. Acta Physiol Scand. 2001;171(2):161-7.
35 Daniele G., Eldor R., Merovci A.*, et al.* Chronic Reduction of Plasma Free Fatty Acid Improves Mitochondrial Function and Whole-Body Insulin Sensitivity in Obese and Type 2 Diabetic Individuals. Diabetes. 2014;63(8):2812-20.
36 Yang G., Li L., Fang C.*, et al.* Effects of Free Fatty Acids on Plasma Resistin and Insulin Resistance in Awake Rats. Metabolism. 2005;54(9):1142-6.
37 Feinstein R., Kanety H., Papa M. Z.*, et al.* Tumor Necrosis Factor-Alpha Suppresses Insulin-Induced Tyrosine Phosphorylation of Insulin Receptor and Its Substrates. J Biol Chem. 1993;268(35):26055-8.
38 Dandona P., Aljada A., Bandyopadhyay A. Inflammation: The Link between Insulin Resistance, Obesity and Diabetes. Trends Immunol. 2004;25(1):4-7.
39 Copps K. D., White M. F. Regulation of Insulin Sensitivity by Serine/Threonine Phosphorylation of Insulin Receptor Substrate Proteins Irs1 and Irs2. Diabetologia. 2012;55(10):2565-82.
40 Tilg H., Moschen A. R. Inflammatory Mechanisms in the Regulation of Insulin Resistance. Mol Med. 2008;14(3-4):222-31.
41 de Luca C., Olefsky J. M. Inflammation and Insulin Resistance. FEBS Lett. 2008;582(1):97-105.
42 Peraldi P., Spiegelman B. Tnf-Alpha and Insulin Resistance: Summary and Future Prospects. Mol Cell Biochem. 1998;182(1-2):169-75.
43 Sifuentes-Franco S., Pacheco-Moisés F. P., Rodríguez-Carrizalez A. D.*, et al.* The Role of Oxidative Stress, Mitochondrial Function, and Autophagy in Diabetic Polyneuropathy. J Diabetes Res. 2017;2017:1673081.
44 Furukawa S., Fujita T., Shimabukuro M.*, et al.* Increased Oxidative Stress in Obesity and Its Impact on Metabolic Syndrome. J Clin Invest. 2004;114(12):1752-61.
45 Giacco F., Brownlee M. Oxidative Stress and Diabetic Complications. Circ Res. 2010;107(9):1058-70.
46 Lin Y., Berg A. H., Iyengar P.*, et al.* The Hyperglycemia-Induced Inflammatory Response in Adipocytes: The Role of Reactive Oxygen Species. J Biol Chem. 2005;280(6):4617-26.
47 Schrauwen-Hinderling V. B., Kooi M. E., Hesselink M. K.*, et al.* Impaired in Vivo Mitochondrial Function but Similar Intramyocellular Lipid Content in Patients with Type 2 Diabetes Mellitus and Bmi-Matched Control Subjects. Diabetologia. 2007;50(1):113-20.
48 Goldstein B. J., Mahadev K., Wu X.*, et al.* Role of Insulin-Induced Reactive Oxygen Species in the Insulin Signaling Pathway. Antioxid Redox Signal. 2005;7(7-8):1021-31.
49 Hurrle S., Hsu W. H. The Etiology of Oxidative Stress in Insulin Resistance. Biomed J. 2017;40(5):257-62.
50 Boyer F., Diotel N., Girard D.*, et al.* Enhanced Oxidative Stress in Adipose Tissue from Diabetic Mice, Possible Contribution of Glycated Albumin. Biochem Biophys Res Commun. 2016;473(1):154-60.
51 Butler A. E., Janson J., Bonner-Weir S.*, et al.* Beta-Cell Deficit and Increased Beta-Cell Apoptosis in Humans with Type 2 Diabetes. Diabetes. 2003;52(1):102-10.
52 Rahier J., Guiot Y., Goebbels R. M.*, et al.* Pancreatic Beta-Cell Mass in European Subjects with Type 2 Diabetes. Diabetes Obes Metab. 2008;10 Suppl 4:32-42.

53 Sakuraba H., Mizukami H., Yagihashi N.*, et al.* Reduced Beta-Cell Mass and Expression of Oxidative Stress-Related DNA Damage in the Islet of Japanese Type Ii Diabetic Patients. Diabetologia. 2002;45(1):85-96.

54 Poitout V., Robertson R. P. Glucolipotoxicity: Fuel Excess and Beta-Cell Dysfunction. Endocr Rev. 2008;29(3):351-66.

55 Poitout V., Robertson R. P. Minireview: Secondary Beta-Cell Failure in Type 2 Diabetes--a Convergence of Glucotoxicity and Lipotoxicity. Endocrinology. 2002;143(2):339-42.

56 Manco M., Bertuzzi A., Salinari S.*, et al.* The Ingestion of Saturated Fatty Acid Triacylglycerols Acutely Affects Insulin Secretion and Insulin Sensitivity in Human Subjects. Br J Nutr. 2004;92(6):895-903.

57 Carpentier A., Mittelman S. D., Lamarche B.*, et al.* Acute Enhancement of Insulin Secretion by Ffa in Humans Is Lost with Prolonged Ffa Elevation. Am J Physiol. 1999;276(6):E1055-66.

58 Kashyap S., Belfort R., Gastaldelli A.*, et al.* A Sustained Increase in Plasma Free Fatty Acids Impairs Insulin Secretion in Nondiabetic Subjects Genetically Predisposed to Develop Type 2 Diabetes. Diabetes. 2003;52(10):2461-74.

59 Weir G. C. Glucolipotoxicity, B-Cells, and Diabetes: The Emperor Has No Clothes. Diabetes. 2020;69(3):273-8.

60 Poitout V., Amyot J., Semache M.*, et al.* Glucolipotoxicity of the Pancreatic Beta Cell. Biochim Biophys Acta. 2010;1801(3):289-98.

61 Kooptiwut S., Kebede M., Zraika S.*, et al.* High Glucose-Induced Impairment in Insulin Secretion Is Associated with Reduction in Islet Glucokinase in a Mouse Model of Susceptibility to Islet Dysfunction. J Mol Endocrinol. 2005;35(1):39-48.

62 Eizirik D. c. L., Jansson L., Flodström M.*, et al.* Mechanisms of Defective Glucose-Induced Insulin Release in Human Pancreatic Islets Transplanted to Diabetic Nude Mice1. The Journal of Clinical Endocrinology & Metabolism. 1997;82(8):2660-3.

63 Solomon T. P. J., Knudsen S. H., Karstoft K.*, et al.* Examining the Effects of Hyperglycemia on Pancreatic Endocrine Function in Humans: Evidence for in Vivo Glucotoxicity. The Journal of Clinical Endocrinology & Metabolism. 2012;97(12):4682-91.

64 Harmon J. S., Gleason C. E., Tanaka Y.*, et al.* In Vivo Prevention of Hyperglycemia Also Prevents Glucotoxic Effects on Pdx-1 and Insulin Gene Expression. Diabetes. 1999;48(10):1995-2000.

65 Ihara Y., Toyokuni S., Uchida K.*, et al.* Hyperglycemia Causes Oxidative Stress in Pancreatic Beta-Cells of Gk Rats, a Model of Type 2 Diabetes. Diabetes. 1999;48(4):927-32.

66 Ishikawa K., Tsunekawa S., Ikeniwa M.*, et al.* Long-Term Pancreatic Beta Cell Exposure to High Levels of Glucose but Not Palmitate Induces DNA Methylation within the Insulin Gene Promoter and Represses Transcriptional Activity. PLoS One. 2015;10(2):e0115350.

67 Mandrup-Poulsen T. Beta-Cell Apoptosis: Stimuli and Signaling. Diabetes. 2001;50 Suppl 1:S58-63.

68 Federici M., Hribal M., Perego L.*, et al.* High Glucose Causes Apoptosis in Cultured Human Pancreatic Islets of Langerhans: A Potential Role for Regulation of Specific Bcl Family Genes toward an Apoptotic Cell Death Program. Diabetes. 2001;50(6):1290-301.

69 Jonas J. C., Sharma A., Hasenkamp W.*, et al.* Chronic Hyperglycemia Triggers Loss of Pancreatic Beta Cell Differentiation in an Animal Model of Diabetes. J Biol Chem. 1999;274(20):14112-21.

70 Laybutt D. R., Glandt M., Xu G.*, et al.* Critical Reduction in Beta-Cell Mass Results in Two Distinct Outcomes over Time. Adaptation with Impaired Glucose Tolerance or Decompensated Diabetes. J Biol Chem. 2003;278(5):2997-3005.

71 Wang Z., York N. W., Nichols C. G.*, et al.* Pancreatic B Cell Dedifferentiation in Diabetes and Redifferentiation Following Insulin Therapy. Cell Metab. 2014;19(5):872-82.

72 Florez J. C., Burtt N., de Bakker P. I.*, et al.* Haplotype Structure and Genotype-Phenotype Correlations of the Sulfonylurea Receptor and the Islet Atp-Sensitive Potassium Channel Gene Region. Diabetes. 2004;53(5):1360-8.

73 Gloyn A. L., Weedon M. N., Owen K. R.*, et al.* Large-Scale Association Studies of Variants in Genes Encoding the Pancreatic Beta-Cell Katp Channel Subunits Kir6.2 (Kcnj11) and Sur1 (Abcc8) Confirm That the Kcnj11 E23k Variant Is Associated with Type 2 Diabetes. Diabetes. 2003;52(2):568-72.

74 Mtiraoui N., Turki A., Nemr R.*, et al.* Contribution of Common Variants of Enpp1, Igf2bp2, Kcnj11, Mlxipl, Pparγ, Slc30a8 and Tcf7l2 to the Risk of Type 2 Diabetes in Lebanese and Tunisian Arabs. Diabetes Metab. 2012;38(5):444-9.

75 Grant S. F., Thorleifsson G., Reynisdottir I.*, et al.* Variant of Transcription Factor 7-Like 2 (Tcf7l2) Gene Confers Risk of Type 2 Diabetes. Nat Genet. 2006;38(3):320-3.

76 Sladek R., Rocheleau G., Rung J.*, et al.* A Genome-Wide Association Study Identifies Novel Risk Loci for Type 2 Diabetes. Nature. 2007;445(7130):881-5.

77 Billings L. K., Florez J. C. The Genetics of Type 2 Diabetes: What Have We Learned from Gwas? Ann N Y Acad Sci. 2010;1212:59-77.

78 World Health Organisation. Obesity and Overweight. 2019.

79 Kelly T., Yang W., Chen C. S.*, et al.* Global Burden of Obesity in 2005 and Projections to 2030. Int J Obes (Lond). 2008;32(9):1431-7.

80 Swinburn B. A., Sacks G., Hall K. D.*, et al.* The Global Obesity Pandemic: Shaped by Global Drivers and Local Environments. Lancet. 2011;378(9793):804-14.

81 Bhurosy T., Jeewon R. Overweight and Obesity Epidemic in Developing Countries: A Problem with Diet, Physical Activity, or Socioeconomic Status? ScientificWorldJournal. 2014;2014:964236.

82 Żukiewicz-Sobczak W., Wróblewska P., Zwoliński J.*, et al.* Obesity and Poverty Paradox in Developed Countries. Ann Agric Environ Med. 2014;21(3):590-4.

83 Prentice A. M. The Emerging Epidemic of Obesity in Developing Countries. Int J Epidemiol. 2006;35(1):93-9.

84 Public Health England. Health Matters: Obesity and the Food Environment 2017 [Available from: https://www.gov.uk/government/publications/health-matters-obesity-and-the-food-environment/health-matters-obesity-and-the-food-environment--2.

85 Bhatnagar A. Environmental Determinants of Cardiovascular Disease. Circ Res. 2017;121(2):162-80.

86 Bjørge T., Häggström C., Ghaderi S.*, et al.* Bmi and Weight Changes and Risk of Obesity-Related Cancers: A Pooled European Cohort Study. Int J Epidemiol. 2019;48(6):1872-85.

87 Anand P., Kunnumakkara A. B., Sundaram C.*, et al.* Cancer Is a Preventable Disease That Requires Major Lifestyle Changes. Pharm Res. 2008;25(9):2097-116.
88 Teras L. R., Patel A. V., Wang M.*, et al.* Sustained Weight Loss and Risk of Breast Cancer in Women ≥50 Years: A Pooled Analysis of Prospective Data. J Natl Cancer Inst. 2019.
89 Gilbert C. A., Slingerland J. M. Cytokines, Obesity, and Cancer: New Insights on Mechanisms Linking Obesity to Cancer Risk and Progression. Annu Rev Med. 2013;64:45-57.
90 Louie S. M., Roberts L. S., Nomura D. K. Mechanisms Linking Obesity and Cancer. Biochim Biophys Acta. 2013;1831(10):1499-508.
91 Unger R. H. Lipid Overload and Overflow: Metabolic Trauma and the Metabolic Syndrome. Trends Endocrinol Metab. 2003;14(9):398-403.
92 Sharma R. B., Alonso L. C. Lipotoxicity in the Pancreatic Beta Cell: Not Just Survival and Function, but Proliferation as Well? Curr Diab Rep. 2014;14(6):492.
93 Qiang G., Whang Kong H., Xu S.*, et al.* Lipodystrophy and Severe Metabolic Dysfunction in Mice with Adipose Tissue-Specific Insulin Receptor Ablation. Mol Metab. 2016;5(7):480-90.
94 Li X., Morita M., Kikuguchi C.*, et al.* Adipocyte-Specific Disruption of Mouse Cnot3 Causes Lipodystrophy. FEBS Lett. 2017;591(2):358-68.
95 Lamont B. J., Waters M. F., Andrikopoulos S. A Low-Carbohydrate High-Fat Diet Increases Weight Gain and Does Not Improve Glucose Tolerance, Insulin Secretion or B-Cell Mass in Nzo Mice. Nutr Diabetes. 2016;6(2):e194.
96 Kluth O., Mirhashemi F., Scherneck S.*, et al.* Dissociation of Lipotoxicity and Glucotoxicity in a Mouse Model of Obesity Associated Diabetes: Role of Forkhead Box O1 (Foxo1) in Glucose-Induced Beta Cell Failure. Diabetologia. 2011;54(3):605-16.
97 Mansoor N., Vinknes K. J., Veierød M. B.*, et al.* Effects of Low-Carbohydrate Diets V. Low-Fat Diets on Body Weight and Cardiovascular Risk Factors: A Meta-Analysis of Randomised Controlled Trials. Br J Nutr. 2016;115(3):466-79.
98 Wang L. L., Wang Q., Hong Y.*, et al.* The Effect of Low-Carbohydrate Diet on Glycemic Control in Patients with Type 2 Diabetes Mellitus. Nutrients. 2018;10(6).
99 Arita Y., Kihara S., Ouchi N.*, et al.* Paradoxical Decrease of an Adipose-Specific Protein, Adiponectin, in Obesity. Biochem Biophys Res Commun. 1999;257(1):79-83.
100 Enomoto T., Ohashi K., Shibata R.*, et al.* Adipolin/C1qdc2/Ctrp12 Protein Functions as an Adipokine That Improves Glucose Metabolism. J Biol Chem. 2011;286(40):34552-8.
101 Chadt A., Scherneck S., Joost H. G.*, et al.* Molecular Links between Obesity and Diabetes: "Diabesity". In: Feingold KR, Anawalt B, Boyce A, Chrousos G, Dungan K, Grossman A, et al., editors. Endotext. South Dartmouth (MA): MDText.com, Inc.
Copyright © 2000-2020, MDText.com, Inc.; 2018.
102 Rosenbaum M., Leibel R. L. 20 Years of Leptin: Role of Leptin in Energy Homeostasis in Humans. J Endocrinol. 2014;223(1):T83-96.
103 Frederich R. C., Hamann A., Anderson S.*, et al.* Leptin Levels Reflect Body Lipid Content in Mice: Evidence for Diet-Induced Resistance to Leptin Action. Nat Med. 1995;1(12):1311-4.
104 Marino J. S., Xu Y., Hill J. W. Central Insulin and Leptin-Mediated Autonomic Control of Glucose Homeostasis. Trends Endocrinol Metab. 2011;22(7):275-85.

105 Rahmouni K., Sigmund C. D., Haynes W. G.*, et al.* Hypothalamic Erk Mediates the Anorectic and Thermogenic Sympathetic Effects of Leptin. Diabetes. 2009;58(3):536-42.

106 Toda C., Shiuchi T., Lee S.*, et al.* Distinct Effects of Leptin and a Melanocortin Receptor Agonist Injected into Medial Hypothalamic Nuclei on Glucose Uptake in Peripheral Tissues. Diabetes. 2009;58(12):2757-65.

107 Fogteloo A. J., Pijl H., Frölich M.*, et al.* Effects of Recombinant Human Leptin Treatment as an Adjunct of Moderate Energy Restriction on Body Weight, Resting Energy Expenditure and Energy Intake in Obese Humans. Diabetes Nutr Metab. 2003;16(2):109-14.

108 Gabriely I., Ma X. H., Yang X. M.*, et al.* Leptin Resistance During Aging Is Independent of Fat Mass. Diabetes. 2002;51(4):1016-21.

109 Dobbins R. L., Chester M. W., Daniels M. B.*, et al.* Circulating Fatty Acids Are Essential for Efficient Glucose-Stimulated Insulin Secretion after Prolonged Fasting in Humans. Diabetes. 1998;47(10):1613-8.

110 Stein D. T., Esser V., Stevenson B. E.*, et al.* Essentiality of Circulating Fatty Acids for Glucose-Stimulated Insulin Secretion in the Fasted Rat. J Clin Invest. 1996;97(12):2728-35.

111 Astiarraga B., Chueire V. B., Souza A. L.*, et al.* Effects of Acute Nefa Manipulation on Incretin-Induced Insulin Secretion in Participants with and without Type 2 Diabetes. Diabetologia. 2018;61(8):1829-37.

112 He W., Yuan T., Maedler K. Macrophage-Associated Pro-Inflammatory State in Human Islets from Obese Individuals. Nutrition & Diabetes. 2019;9(1):36.

113 Oh D. Y., Morinaga H., Talukdar S.*, et al.* Increased Macrophage Migration into Adipose Tissue in Obese Mice. Diabetes. 2012;61(2):346-54.

114 Patsouris D., Cao J. J., Vial G.*, et al.* Insulin Resistance Is Associated with Mcp1-Mediated Macrophage Accumulation in Skeletal Muscle in Mice and Humans. PLoS One. 2014;9(10):e110653.

115 Wentworth J. M., Naselli G., Brown W. A.*, et al.* Pro-Inflammatory Cd11c+Cd206+ Adipose Tissue Macrophages Are Associated with Insulin Resistance in Human Obesity. Diabetes. 2010;59(7):1648-56.

116 Arango Duque G., Descoteaux A. Macrophage Cytokines: Involvement in Immunity and Infectious Diseases. Front Immunol. 2014;5:491.

117 Krogh-Madsen R., Plomgaard P., Møller K.*, et al.* Influence of Tnf-Alpha and Il-6 Infusions on Insulin Sensitivity and Expression of Il-18 in Humans. Am J Physiol Endocrinol Metab. 2006;291(1):E108-14.

118 Senn J. J., Klover P. J., Nowak I. A.*, et al.* Interleukin-6 Induces Cellular Insulin Resistance in Hepatocytes. Diabetes. 2002;51(12):3391-9.

119 Narayan K. M., Boyle J. P., Thompson T. J.*, et al.* Effect of Bmi on Lifetime Risk for Diabetes in the U.S. Diabetes Care. 2007;30(6):1562-6.

120 Langenberg C., Sharp S. J., Schulze M. B.*, et al.* Long-Term Risk of Incident Type 2 Diabetes and Measures of Overall and Regional Obesity: The Epic-Interact Case-Cohort Study. PLoS Med. 2012;9(6):e1001230.

121 Ganz M. L., Wintfeld N., Li Q.*, et al.* The Association of Body Mass Index with the Risk of Type 2 Diabetes: A Case-Control Study Nested in an Electronic Health Records System in the United States. Diabetol Metab Syndr. 2014;6(1):50.

122 Gray N., Picone G., Sloan F.*, et al.* Relation between Bmi and Diabetes Mellitus and Its Complications among Us Older Adults. South Med J. 2015;108(1):29-36.
123 Hu F. B., Manson J. E., Stampfer M. J.*, et al.* Diet, Lifestyle, and the Risk of Type 2 Diabetes Mellitus in Women. N Engl J Med. 2001;345(11):790-7.
124 Steinbrecher A., Erber E., Grandinetti A.*, et al.* Meat Consumption and Risk of Type 2 Diabetes: The Multiethnic Cohort. Public Health Nutr. 2011;14(4):568-74.
125 Wilding J. P. The Importance of Weight Management in Type 2 Diabetes Mellitus. Int J Clin Pract. 2014;68(6):682-91.
126 Forouhi N. G., Misra A., Mohan V.*, et al.* Dietary and Nutritional Approaches for Prevention and Management of Type 2 Diabetes. BMJ. 2018;361:k2234.
127 Kim E. S., Jeong J. S., Han K.*, et al.* Impact of Weight Changes on the Incidence of Diabetes Mellitus: A Korean Nationwide Cohort Study. Scientific Reports. 2018;8(1):3735.
128 Whitmore C. Type 2 Diabetes and Obesity in Adults. Br J Nurs. 2010;19(14):880, 2-6.
129 Gower B. A., Weinsier R. L., Jordan J. M.*, et al.* Effects of Weight Loss on Changes in Insulin Sensitivity and Lipid Concentrations in Premenopausal African American and White Women. Am J Clin Nutr. 2002;76(5):923-7.
130 Houmard J. A., Tanner C. J., Yu C.*, et al.* Effect of Weight Loss on Insulin Sensitivity and Intramuscular Long-Chain Fatty Acyl-Coas in Morbidly Obese Subjects. Diabetes. 2002;51(10):2959-63.
131 Schenk S., Harber M. P., Shrivastava C. R.*, et al.* Improved Insulin Sensitivity after Weight Loss and Exercise Training Is Mediated by a Reduction in Plasma Fatty Acid Mobilization, Not Enhanced Oxidative Capacity. J Physiol. 2009;587(Pt 20):4949-61.
132 Clamp L. D., Hume D. J., Lambert E. V.*, et al.* Enhanced Insulin Sensitivity in Successful, Long-Term Weight Loss Maintainers Compared with Matched Controls with No Weight Loss History. Nutr Diabetes. 2017;7(6):e282.
133 Knowler W. C., Barrett-Connor E., Fowler S. E.*, et al.* Reduction in the Incidence of Type 2 Diabetes with Lifestyle Intervention or Metformin. N Engl J Med. 2002;346(6):393-403.
134 Knowler W. C., Fowler S. E., Hamman R. F.*, et al.* 10-Year Follow-up of Diabetes Incidence and Weight Loss in the Diabetes Prevention Program Outcomes Study. Lancet. 2009;374(9702):1677-86.
135 Yang S., Wang S., Yang B.*, et al.* Weight Loss before a Diagnosis of Type 2 Diabetes Mellitus Is a Risk Factor for Diabetes Complications. Medicine (Baltimore). 2016;95(49):e5618.
136 Resnick H. E., Valsania P., Halter J. B.*, et al.* Relation of Weight Gain and Weight Loss on Subsequent Diabetes Risk in Overweight Adults. J Epidemiol Community Health. 2000;54(8):596-602.
137 Wu T., Gao X., Chen M.*, et al.* Long-Term Effectiveness of Diet-Plus-Exercise Interventions Vs. Diet-Only Interventions for Weight Loss: A Meta-Analysis. Obes Rev. 2009;10(3):313-23.
138 Wing R. R., Phelan S. Long-Term Weight Loss Maintenance. Am J Clin Nutr. 2005;82(1 Suppl):222s-5s.
139 Loveman E., Frampton G. K., Shepherd J.*, et al.* The Clinical Effectiveness and Cost-Effectiveness of Long-Term Weight Management Schemes for Adults: A Systematic Review. Health Technol Assess. 2011;15(2):1-182.

140 Donner T., Sarkar S. Insulin – Pharmacology, Therapeutic Regimens, and Principles of Intensive Insulin Therapy. In: Feingold KR, Anawalt B, Boyce A, Chrousos G, Dungan K, Grossman A, et al., editors. Endotext. South Dartmouth (MA): MDText.com, Inc.
Copyright © 2000-2020, MDText.com, Inc.; 2000.

141 Chon S., Oh S., Kim S. W.*, et al.* The Effect of Early Insulin Therapy on Pancreatic B-Cell Function and Long-Term Glycemic Control in Newly Diagnosed Type 2 Diabetic Patients. Korean J Intern Med. 2010;25(3):273-81.

142 Hu Y., Li L., Xu Y.*, et al.* Short-Term Intensive Therapy in Newly Diagnosed Type 2 Diabetes Partially Restores Both Insulin Sensitivity and B-Cell Function in Subjects with Long-Term Remission. Diabetes Care. 2011;34(8):1848-53.

143 Weng J., Li Y., Xu W.*, et al.* Effect of Intensive Insulin Therapy on Beta-Cell Function and Glycaemic Control in Patients with Newly Diagnosed Type 2 Diabetes: A Multicentre Randomised Parallel-Group Trial. Lancet. 2008;371(9626):1753-60.

144 Chen H. S., Wu T. E., Kuo C. S. Long-Term Glycemic Control after 6 Months of Basal Insulin Therapy. Am J Manag Care. 2014;20(9):e369-79.

145 Ilkova H., Glaser B., Tunçkale A.*, et al.* Induction of Long-Term Glycemic Control in Newly Diagnosed Type 2 Diabetic Patients by Transient Intensive Insulin Treatment. Diabetes Care. 1997;20(9):1353-6.

146 Skovsø S., Damgaard J., Fels J. J.*, et al.* Effects of Insulin Therapy on Weight Gain and Fat Distribution in the Hf/Hs-Stz Rat Model of Type 2 Diabetes. International Journal of Obesity. 2015;39(10):1531-8.

147 Ter Braak B., Siezen C. L., Lee J. S.*, et al.* Insulin-Like Growth Factor 1 Receptor Activation Promotes Mammary Gland Tumor Development by Increasing Glycolysis and Promoting Biomass Production. Breast Cancer Res. 2017;19(1):14.

148 Lamanna C., Monami M., Marchionni N.*, et al.* Effect of Metformin on Cardiovascular Events and Mortality: A Meta-Analysis of Randomized Clinical Trials. Diabetes Obes Metab. 2011;13(3):221-8.

149 Palmer S. C., Strippoli G. F. M. Metformin as First-Line Treatment for Type 2 Diabetes. Lancet. 2018;392(10142):120.

150 Rena G., Hardie D. G., Pearson E. R. The Mechanisms of Action of Metformin. Diabetologia. 2017;60(9):1577-85.

151 Féry F., Plat L., Balasse E. O. Effects of Metformin on the Pathways of Glucose Utilization after Oral Glucose in Non-Insulin-Dependent Diabetes Mellitus Patients. Metabolism. 1997;46(2):227-33.

152 Garber A. J., Duncan T. G., Goodman A. M.*, et al.* Efficacy of Metformin in Type Ii Diabetes: Results of a Double-Blind, Placebo-Controlled, Dose-Response Trial. Am J Med. 1997;103(6):491-7.

153 Ong C. R., Molyneaux L. M., Constantino M. I.*, et al.* Long-Term Efficacy of Metformin Therapy in Nonobese Individuals with Type 2 Diabetes. Diabetes Care. 2006;29(11):2361-4.

154 Ito H., Ishida H., Takeuchi Y.*, et al.* Long-Term Effect of Metformin on Blood Glucose Control in Non-Obese Patients with Type 2 Diabetes Mellitus. Nutr Metab (Lond). 2010;7:83.

155 DeFronzo R. A., Goodman A. M. Efficacy of Metformin in Patients with Non-Insulin-Dependent Diabetes Mellitus. The Multicenter Metformin Study Group. N Engl J Med. 1995;333(9):541-9.

156 Hoffmann J., Spengler M. Efficacy of 24-Week Monotherapy with Acarbose, Metformin, or Placebo in Dietary-Treated Niddm Patients: The Essen-Ii Study. Am J Med. 1997;103(6):483-90.

157 Horton E. S., Clinkingbeard C., Gatlin M.*, et al.* Nateglinide Alone and in Combination with Metformin Improves Glycemic Control by Reducing Mealtime Glucose Levels in Type 2 Diabetes. Diabetes Care. 2000;23(11):1660-5.

158 Fujioka K., Brazg R. L., Raz I.*, et al.* Efficacy, Dose-Response Relationship and Safety of Once-Daily Extended-Release Metformin (Glucophage Xr) in Type 2 Diabetic Patients with Inadequate Glycaemic Control Despite Prior Treatment with Diet and Exercise: Results from Two Double-Blind, Placebo-Controlled Studies. Diabetes Obes Metab. 2005;7(1):28-39.

159 Chiasson J. L., Naditch L. The Synergistic Effect of Miglitol Plus Metformin Combination Therapy in the Treatment of Type 2 Diabetes. Diabetes Care. 2001;24(6):989-94.

160 Goldstein B. J., Feinglos M. N., Lunceford J. K.*, et al.* Effect of Initial Combination Therapy with Sitagliptin, a Dipeptidyl Peptidase-4 Inhibitor, and Metformin on Glycemic Control in Patients with Type 2 Diabetes. Diabetes Care. 2007;30(8):1979-87.

161 van der Aa M. P., Elst M. A., van de Garde E. M.*, et al.* Long-Term Treatment with Metformin in Obese, Insulin-Resistant Adolescents: Results of a Randomized Double-Blinded Placebo-Controlled Trial. Nutr Diabetes. 2016;6(8):e228.

162 Strowig S. M., Avilés-Santa M. L., Raskin P. Comparison of Insulin Monotherapy and Combination Therapy with Insulin and Metformin or Insulin and Troglitazone in Type 2 Diabetes. Diabetes Care. 2002;25(10):1691-8.

163 Tosi F., Muggeo M., Brun E.*, et al.* Combination Treatment with Metformin and Glibenclamide Versus Single-Drug Therapies in Type 2 Diabetes Mellitus: A Randomized, Double-Blind, Comparative Study. Metabolism. 2003;52(7):862-7.

164 Charbonnel B., Schernthaner G., Brunetti P.*, et al.* Long-Term Efficacy and Tolerability of Add-on Pioglitazone Therapy to Failing Monotherapy Compared with Addition of Gliclazide or Metformin in Patients with Type 2 Diabetes. Diabetologia. 2005;48(6):1093-104.

165 Hanefeld M., Brunetti P., Schernthaner G. H.*, et al.* One-Year Glycemic Control with a Sulfonylurea Plus Pioglitazone Versus a Sulfonylurea Plus Metformin in Patients with Type 2 Diabetes. Diabetes Care. 2004;27(1):141-7.

166 Henry R. R., Murray A. V., Marmolejo M. H.*, et al.* Dapagliflozin, Metformin Xr, or Both: Initial Pharmacotherapy for Type 2 Diabetes, a Randomised Controlled Trial. Int J Clin Pract. 2012;66(5):446-56.

167 Hadjadj S., Rosenstock J., Meinicke T.*, et al.* Initial Combination of Empagliflozin and Metformin in Patients with Type 2 Diabetes. Diabetes Care. 2016;39(10):1718-28.

168 Rosenstock J., Chuck L., González-Ortiz M.*, et al.* Initial Combination Therapy with Canagliflozin Plus Metformin Versus Each Component as Monotherapy for Drug-Naïve Type 2 Diabetes. Diabetes Care. 2016;39(3):353-62.

169 Choudhury S., Hirschberg Y., Filipek R.*, et al.* Single-Dose Pharmacokinetics of Nateglinide in Subjects with Hepatic Cirrhosis. J Clin Pharmacol. 2000;40(6):634-40.

170 Cho Y. M., Koo B. K., Son H. Y.*, et al.* Effect of the Combination of Mitiglinide and Metformin on Glycemic Control in Patients with Type 2 Diabetes Mellitus. J Diabetes Investig. 2010;1(4):143-8.

171 Inzucchi S. E., Maggs D. G., Spollett G. R.*, et al.* Efficacy and Metabolic Effects of Metformin and Troglitazone in Type Ii Diabetes Mellitus. N Engl J Med. 1998;338(13):867-72.
172 Stumvoll M., Nurjhan N., Perriello G.*, et al.* Metabolic Effects of Metformin in Non-Insulin-Dependent Diabetes Mellitus. N Engl J Med. 1995;333(9):550-4.
173 Lee A., Morley J. E. Metformin Decreases Food Consumption and Induces Weight Loss in Subjects with Obesity with Type Ii Non-Insulin-Dependent Diabetes. Obes Res. 1998;6(1):47-53.
174 Long-Term Safety, Tolerability, and Weight Loss Associated with Metformin in the Diabetes Prevention Program Outcomes Study. Diabetes Care. 2012;35(4):731-7.
175 Seifarth C., Schehler B., Schneider H. J. Effectiveness of Metformin on Weight Loss in Non-Diabetic Individuals with Obesity. Exp Clin Endocrinol Diabetes. 2013;121(1):27-31.
176 Grant P. J. The Effects of High- and Medium-Dose Metformin Therapy on Cardiovascular Risk Factors in Patients with Type Ii Diabetes. Diabetes Care. 1996;19(1):64-6.
177 Ashcroft F. M. Mechanisms of the Glycaemic Effects of Sulfonylureas. Horm Metab Res. 1996;28(9):456-63.
178 Guardado-Mendoza R., Prioletta A., Jiménez-Ceja L. M.*, et al.* The Role of Nateglinide and Repaglinide, Derivatives of Meglitinide, in the Treatment of Type 2 Diabetes Mellitus. Arch Med Sci. 2013;9(5):936-43.
179 Grant J. S., Graven L. J. Progressing from Metformin to Sulfonylureas or Meglitinides. Workplace Health Saf. 2016;64(9):433-9.
180 Mearns E. S., Sobieraj D. M., White C. M.*, et al.* Comparative Efficacy and Safety of Antidiabetic Drug Regimens Added to Metformin Monotherapy in Patients with Type 2 Diabetes: A Network Meta-Analysis. PLoS One. 2015;10(4):e0125879.
181 Mishriky B. M., Cummings D. M., Tanenberg R. J. The Efficacy and Safety of Dpp4 Inhibitors Compared to Sulfonylureas as Add-on Therapy to Metformin in Patients with Type 2 Diabetes: A Systematic Review and Meta-Analysis. Diabetes Res Clin Pract. 2015;109(2):378-88.
182 Pfeiffer A. F., Klein H. H. The Treatment of Type 2 Diabetes. Dtsch Arztebl Int. 2014;111(5):69-81; quiz 2.
183 Azoulay L., Suissa S. Sulfonylureas and the Risks of Cardiovascular Events and Death: A Methodological Meta-Regression Analysis of the Observational Studies. Diabetes Care. 2017;40(5):706-14.
184 Kalra S., Bahendeka S., Sahay R.*, et al.* Consensus Recommendations on Sulfonylurea and Sulfonylurea Combinations in the Management of Type 2 Diabetes Mellitus - International Task Force. Indian J Endocrinol Metab. 2018;22(1):132-57.
185 Hauner H. The Mode of Action of Thiazolidinediones. Diabetes Metab Res Rev. 2002;18 Suppl 2:S10-5.
186 Fonseca V., Rosenstock J., Patwardhan R.*, et al.* Effect of Metformin and Rosiglitazone Combination Therapy in Patients with Type 2 Diabetes Mellitus: A Randomized Controlled Trial. Jama. 2000;283(13):1695-702.
187 Kirk J. K., Pearce K. A., Michielutte R.*, et al.* Troglitazone or Metformin in Combination with Sulfonylureas for Patients with Type 2 Diabetes? J Fam Pract. 1999;48(11):879-82.

188 Virtanen K. A., Hällsten K., Parkkola R.*, et al.* Differential Effects of Rosiglitazone and Metformin on Adipose Tissue Distribution and Glucose Uptake in Type 2 Diabetic Subjects. Diabetes. 2003;52(2):283-90.

189 Yu J. G., Kruszynska Y. T., Mulford M. I.*, et al.* A Comparison of Troglitazone and Metformin on Insulin Requirements in Euglycemic Intensively Insulin-Treated Type 2 Diabetic Patients. Diabetes. 1999;48(12):2414-21.

190 Hanefeld M. Pioglitazone and Sulfonylureas: Effectively Treating Type 2 Diabetes. Int J Clin Pract Suppl. 2007;61(153):20-7.

191 Horton E. S., Whitehouse F., Ghazzi M. N.*, et al.* Troglitazone in Combination with Sulfonylurea Restores Glycemic Control in Patients with Type 2 Diabetes. The Troglitazone Study Group. Diabetes Care. 1998;21(9):1462-9.

192 Iwamoto Y., Kosaka K., Kuzuya T.*, et al.* Effect of Combination Therapy of Troglitazone and Sulphonylureas in Patients with Type 2 Diabetes Who Were Poorly Controlled by Sulphonylurea Therapy Alone. Diabet Med. 1996;13(4):365-70.

193 Kaku K., Hashiramoto M. Thiazolidinediones and Bone Fractures. J Diabetes Investig. 2011;2(5):354-5.

194 Nesto R. W., Bell D., Bonow R. O.*, et al.* Thiazolidinedione Use, Fluid Retention, and Congestive Heart Failure: A Consensus Statement from the American Heart Association and American Diabetes Association. Diabetes Care. 2004;27(1):256-63.

195 Nissen S. E., Wolski K. Effect of Rosiglitazone on the Risk of Myocardial Infarction and Death from Cardiovascular Causes. N Engl J Med. 2007;356(24):2457-71.

196 Wilding J. Thiazolidinediones, Insulin Resistance and Obesity: Finding a Balance. Int J Clin Pract. 2006;60(10):1272-80.

197 Brown E., Rajeev S. P., Cuthbertson D. J.*, et al.* A Review of the Mechanism of Action, Metabolic Profile and Haemodynamic Effects of Sodium-Glucose Co-Transporter-2 Inhibitors. Diabetes Obes Metab. 2019;21 Suppl 2:9-18.

198 Rossetti L., Smith D., Shulman G. I.*, et al.* Correction of Hyperglycemia with Phlorizin Normalizes Tissue Sensitivity to Insulin in Diabetic Rats. J Clin Invest. 1987;79(5):1510-5.

199 Maliha G., Townsend R. R. Sglt2 Inhibitors: Their Potential Reduction in Blood Pressure. J Am Soc Hypertens. 2015;9(1):48-53.

200 Kawasoe S., Maruguchi Y., Kajiya S.*, et al.* Mechanism of the Blood Pressure-Lowering Effect of Sodium-Glucose Cotransporter 2 Inhibitors in Obese Patients with Type 2 Diabetes. BMC Pharmacol Toxicol. 2017;18(1):23.

201 Brown E., Wilding J. P. H., Barber T. M.*, et al.* Weight Loss Variability with Sglt2 Inhibitors and Glp-1 Receptor Agonists in Type 2 Diabetes Mellitus and Obesity: Mechanistic Possibilities. Obes Rev. 2019;20(6):816-28.

202 Ribola F. A., Cançado F. B., Schoueri J. H.*, et al.* Effects of Sglt2 Inhibitors on Weight Loss in Patients with Type 2 Diabetes Mellitus. Eur Rev Med Pharmacol Sci. 2017;21(1):199-211.

203 Li J., Gong Y., Li C.*, et al.* Long-Term Efficacy and Safety of Sodium-Glucose Cotransporter-2 Inhibitors as Add-on to Metformin Treatment in the Management of Type 2 Diabetes Mellitus: A Meta-Analysis. Medicine (Baltimore). 2017;96(27):e7201.

204 Buchwald H., Avidor Y., Braunwald E.*, et al.* Bariatric Surgery: A Systematic Review and Meta-Analysis. Jama. 2004;292(14):1724-37.

205 Park C. H., Nam S. J., Choi H. S.*, et al.* Comparative Efficacy of Bariatric Surgery in the Treatment of Morbid Obesity and Diabetes Mellitus: A Systematic Review and Network Meta-Analysis. Obes Surg. 2019;29(7):2180-90.

206 Buchwald H., Estok R., Fahrbach K.*, et al.* Trends in Mortality in Bariatric Surgery: A Systematic Review and Meta-Analysis. Surgery. 2007;142(4):621-32; discussion 32-5.

207 National Institute for Health and Care Excellence. Obesity: Identification, Assessment and Management 2014 [Available from: https://www.nice.org.uk/guidance/cg189/chapter/1-recommendations.

208 Astiarraga B., Gastaldelli A., Muscelli E.*, et al.* Biliopancreatic Diversion in Nonobese Patients with Type 2 Diabetes: Impact and Mechanisms. J Clin Endocrinol Metab. 2013;98(7):2765-73.

209 Batterham R. L., Cummings D. E. Mechanisms of Diabetes Improvement Following Bariatric/Metabolic Surgery. Diabetes Care. 2016;39(6):893-901.

210 Baskota A., Li S., Dhakal N.*, et al.* Bariatric Surgery for Type 2 Diabetes Mellitus in Patients with Bmi <30 Kg/M2: A Systematic Review and Meta-Analysis. PLoS One. 2015;10(7):e0132335.

211 Rao W. S., Shan C. X., Zhang W.*, et al.* A Meta-Analysis of Short-Term Outcomes of Patients with Type 2 Diabetes Mellitus and Bmi ≤ 35 Kg/M2 Undergoing Roux-En-Y Gastric Bypass. World J Surg. 2015;39(1):223-30.

212 Pories W. J., Swanson M. S., MacDonald K. G.*, et al.* Who Would Have Thought It? An Operation Proves to Be the Most Effective Therapy for Adult-Onset Diabetes Mellitus. Ann Surg. 1995;222(3):339-50; discussion 50-2.

213 Guidone C., Manco M., Valera-Mora E.*, et al.* Mechanisms of Recovery from Type 2 Diabetes after Malabsorptive Bariatric Surgery. Diabetes. 2006;55(7):2025-31.

214 Chaudhry S., Bernardes M., Harris P. E.*, et al.* Gastrointestinal Dopamine as an Anti-Incretin and Its Possible Role in Bypass Surgery as Therapy for Type 2 Diabetes with Associated Obesity. Minerva Endocrinol. 2016;41(1):43-56.

215 Penney N. C., Kinross J., Newton R. C.*, et al.* The Role of Bile Acids in Reducing the Metabolic Complications of Obesity after Bariatric Surgery: A Systematic Review. Int J Obes (Lond). 2015;39(11):1565-74.

216 Fang S., Suh J. M., Reilly S. M.*, et al.* Intestinal Fxr Agonism Promotes Adipose Tissue Browning and Reduces Obesity and Insulin Resistance. Nat Med. 2015;21(2):159-65.

217 Liou A. P., Paziuk M., Luevano J. M., Jr.*, et al.* Conserved Shifts in the Gut Microbiota Due to Gastric Bypass Reduce Host Weight and Adiposity. Sci Transl Med. 2013;5(178):178ra41.

218 Behary P., Tharakan G., Alexiadou K.*, et al.* Combined Glp-1, Oxyntomodulin, and Peptide Yy Improves Body Weight and Glycemia in Obesity and Prediabetes/Type 2 Diabetes: A Randomized, Single-Blinded, Placebo-Controlled Study. Diabetes Care. 2019;42(8):1446-53.

219 Tan T., Behary P., Tharakan G.*, et al.* The Effect of a Subcutaneous Infusion of Glp-1, Oxm, and Pyy on Energy Intake and Expenditure in Obese Volunteers. J Clin Endocrinol Metab. 2017;102(7):2364-72.

220 Laferrère B. Effect of Gastric Bypass Surgery on the Incretins. Diabetes Metab. 2009;35(6 Pt 2):513-7.

221 Laferrère B., Swerdlow N., Bawa B.*, et al.* Rise of Oxyntomodulin in Response to Oral Glucose after Gastric Bypass Surgery in Patients with Type 2 Diabetes. J Clin Endocrinol Metab. 2010;95(8):4072-6.

222 Mirahmadian M., Hasani M., Taheri E.*, et al.* Influence of Gastric Bypass Surgery on Resting Energy Expenditure, Body Composition, Physical Activity, and Thyroid Hormones in Morbidly Obese Patients. Diabetes Metab Syndr Obes. 2018;11:667-72.
223 Holst J. J., Knop F. K., Vilsbøll T.*, et al.* Loss of Incretin Effect Is a Specific, Important, and Early Characteristic of Type 2 Diabetes. Diabetes Care. 2011;34 Suppl 2(Suppl 2):S251-7.
224 Knop F. K., Vilsbøll T., Højberg P. V.*, et al.* Reduced Incretin Effect in Type 2 Diabetes: Cause or Consequence of the Diabetic State? Diabetes. 2007;56(8):1951-9.
225 Meier J. J., Nauck M. A. Is the Diminished Incretin Effect in Type 2 Diabetes Just an Epi-Phenomenon of Impaired Beta-Cell Function? Diabetes. 2010;59(5):1117-25.
226 Yousseif A., Emmanuel J., Karra E.*, et al.* Differential Effects of Laparoscopic Sleeve Gastrectomy and Laparoscopic Gastric Bypass on Appetite, Circulating Acyl-Ghrelin, Peptide Yy3-36 and Active Glp-1 Levels in Non-Diabetic Humans. Obes Surg. 2014;24(2):241-52.
227 Cegla J., Troke R. C., Jones B.*, et al.* Coinfusion of Low-Dose Glp-1 and Glucagon in Man Results in a Reduction in Food Intake. Diabetes. 2014;63(11):3711-20.
228 Tan T. M., Field B. C., McCullough K. A.*, et al.* Coadministration of Glucagon-Like Peptide-1 During Glucagon Infusion in Humans Results in Increased Energy Expenditure and Amelioration of Hyperglycemia. Diabetes. 2013;62(4):1131-8.
229 Chia C. W., Carlson O. D., Kim W.*, et al.* Exogenous Glucose-Dependent Insulinotropic Polypeptide Worsens Post Prandial Hyperglycemia in Type 2 Diabetes. Diabetes. 2009;58(6):1342-9.
230 Jorsal T., Rhee N. A., Pedersen J.*, et al.* Enteroendocrine K and L Cells in Healthy and Type 2 Diabetic Individuals. Diabetologia. 2018;61(2):284-94.
231 Schroeder W. T., Lopez L. C., Harper M. E.*, et al.* Localization of the Human Glucagon Gene (Gcg) to Chromosome Segment 2q36----37. Cytogenet Cell Genet. 1984;38(1):76-9.
232 Tricoli J. V., Bell G. I., Shows T. B. The Human Glucagon Gene Is Located on Chromosome 2. Diabetes. 1984;33(2):200-2.
233 Holst J. J. The Physiology of Glucagon-Like Peptide 1. Physiol Rev. 2007;87(4):1409-39.
234 Mojsov S., Weir G. C., Habener J. F. Insulinotropin: Glucagon-Like Peptide I (7-37) Co-Encoded in the Glucagon Gene Is a Potent Stimulator of Insulin Release in the Perfused Rat Pancreas. J Clin Invest. 1987;79(2):616-9.
235 Cantini G., Di Franco A., Mannucci E.*, et al.* Is Cleaved Glucagon-Like Peptide 1 Really Inactive? Effects of Glp-1(9-36) on Human Adipose Stem Cells. Mol Cell Endocrinol. 2017;439:10-5.
236 Meier J. J., Gethmann A., Nauck M. A.*, et al.* The Glucagon-Like Peptide-1 Metabolite Glp-1-(9-36) Amide Reduces Postprandial Glycemia Independently of Gastric Emptying and Insulin Secretion in Humans. Am J Physiol Endocrinol Metab. 2006;290(6):E1118-23.
237 Vilsbøll T., Agersø H., Krarup T.*, et al.* Similar Elimination Rates of Glucagon-Like Peptide-1 in Obese Type 2 Diabetic Patients and Healthy Subjects. J Clin Endocrinol Metab. 2003;88(1):220-4.
238 Baggio L. L., Yusta B., Mulvihill E. E.*, et al.* Glp-1 Receptor Expression within the Human Heart. Endocrinology. 2018;159(4):1570-84.

239 Bullock B. P., Heller R. S., Habener J. F. Tissue Distribution of Messenger Ribonucleic Acid Encoding the Rat Glucagon-Like Peptide-1 Receptor. Endocrinology. 1996;137(7):2968-78.

240 Jensen C. B., Pyke C., Rasch M. G.*, et al.* Characterization of the Glucagonlike Peptide-1 Receptor in Male Mouse Brain Using a Novel Antibody and in Situ Hybridization. Endocrinology. 2018;159(2):665-75.

241 Thorens B. Expression Cloning of the Pancreatic Beta Cell Receptor for the Gluco-Incretin Hormone Glucagon-Like Peptide 1. Proc Natl Acad Sci U S A. 1992;89(18):8641-5.

242 Varin E. M., Mulvihill E. E., Baggio L. L.*, et al.* Distinct Neural Sites of Glp-1r Expression Mediate Physiological Versus Pharmacological Control of Incretin Action. Cell Rep. 2019;27(11):3371-84.e3.

243 Edwards C. M., Todd J. F., Mahmoudi M.*, et al.* Glucagon-Like Peptide 1 Has a Physiological Role in the Control of Postprandial Glucose in Humans: Studies with the Antagonist Exendin 9-39. Diabetes. 1999;48(1):86-93.

244 Schirra J., Nicolaus M., Roggel R.*, et al.* Endogenous Glucagon-Like Peptide 1 Controls Endocrine Pancreatic Secretion and Antro-Pyloro-Duodenal Motility in Humans. Gut. 2006;55(2):243-51.

245 Berthoud H. R., Neuhuber W. L. Functional and Chemical Anatomy of the Afferent Vagal System. Auton Neurosci. 2000;85(1-3):1-17.

246 Nakagawa A., Satake H., Nakabayashi H.*, et al.* Receptor Gene Expression of Glucagon-Like Peptide-1, but Not Glucose-Dependent Insulinotropic Polypeptide, in Rat Nodose Ganglion Cells. Auton Neurosci. 2004;110(1):36-43.

247 Krieger J. P., Arnold M., Pettersen K. G.*, et al.* Knockdown of Glp-1 Receptors in Vagal Afferents Affects Normal Food Intake and Glycemia. Diabetes. 2016;65(1):34-43.

248 Lamont B. J., Li Y., Kwan E.*, et al.* Pancreatic Glp-1 Receptor Activation Is Sufficient for Incretin Control of Glucose Metabolism in Mice. J Clin Invest. 2012;122(1):388-402.

249 Kjems L. L., Holst J. J., Vølund A.*, et al.* The Influence of Glp-1 on Glucose-Stimulated Insulin Secretion: Effects on Beta-Cell Sensitivity in Type 2 and Nondiabetic Subjects. Diabetes. 2003;52(2):380-6.

250 Toft-Nielsen M. B., Madsbad S., Holst J. J. Continuous Subcutaneous Infusion of Glucagon-Like Peptide 1 Lowers Plasma Glucose and Reduces Appetite in Type 2 Diabetic Patients. Diabetes Care. 1999;22(7):1137-43.

251 Rosenbaum D. M., Rasmussen S. G., Kobilka B. K. The Structure and Function of G-Protein-Coupled Receptors. Nature. 2009;459(7245):356-63.

252 Jazayeri A., Rappas M., Brown A. J. H.*, et al.* Crystal Structure of the Glp-1 Receptor Bound to a Peptide Agonist. Nature. 2017;546(7657):254-8.

253 Underwood C. R., Garibay P., Knudsen L. B.*, et al.* Crystal Structure of Glucagon-Like Peptide-1 in Complex with the Extracellular Domain of the Glucagon-Like Peptide-1 Receptor. J Biol Chem. 2010;285(1):723-30.

254 Wootten D., Reynolds C. A., Smith K. J.*, et al.* The Extracellular Surface of the Glp-1 Receptor Is a Molecular Trigger for Biased Agonism. Cell. 2016;165(7):1632-43.

255 Zhang Y., Sun B., Feng D.*, et al.* Cryo-Em Structure of the Activated Glp-1 Receptor in Complex with a G Protein. Nature. 2017;546(7657):248-53.

256 Light P. E., Manning Fox J. E., Riedel M. J.*, et al.* Glucagon-Like Peptide-1 Inhibits Pancreatic Atp-Sensitive Potassium Channels Via a Protein Kinase a- and Adp-Dependent Mechanism. Mol Endocrinol. 2002;16(9):2135-44.

257 Kang G., Chepurny O. G., Malester B.*, et al.* Camp Sensor Epac as a Determinant of Atp-Sensitive Potassium Channel Activity in Human Pancreatic Beta Cells and Rat Ins-1 Cells. J Physiol. 2006;573(Pt 3):595-609.

258 Kang G., Leech C. A., Chepurny O. G.*, et al.* Role of the Camp Sensor Epac as a Determinant of Katp Channel Atp Sensitivity in Human Pancreatic Beta-Cells and Rat Ins-1 Cells. J Physiol. 2008;586(5):1307-19.

259 Dzhura I., Chepurny O. G., Kelley G. G.*, et al.* Epac2-Dependent Mobilization of Intracellular Ca^{2}+ by Glucagon-Like Peptide-1 Receptor Agonist Exendin-4 Is Disrupted in B-Cells of Phospholipase C-E Knockout Mice. J Physiol. 2010;588(Pt 24):4871-89.

260 Leech C. A., Chepurny O. G., Holz G. G. Epac2-Dependent Rap1 Activation and the Control of Islet Insulin Secretion by Glucagon-Like Peptide-1. Vitam Horm. 2010;84:279-302.

261 Sasaki S., Miyatsuka T., Matsuoka T. A.*, et al.* Activation of Glp-1 and Gastrin Signalling Induces in Vivo Reprogramming of Pancreatic Exocrine Cells into Beta Cells in Mice. Diabetologia. 2015;58(11):2582-91.

262 Stephens L. R., Eguinoa A., Erdjument-Bromage H.*, et al.* The G Beta Gamma Sensitivity of a Pi3k Is Dependent Upon a Tightly Associated Adaptor, P101. Cell. 1997;89(1):105-14.

263 Yang L., Yao D., Yang H.*, et al.* Puerarin Protects Pancreatic B-Cells in Obese Diabetic Mice Via Activation of Glp-1r Signaling. Mol Endocrinol. 2016;30(3):361-71.

264 Zhang Z., Hu Y., Xu N.*, et al.* A New Way for Beta Cell Neogenesis: Transdifferentiation from Alpha Cells Induced by Glucagon-Like Peptide 1. J Diabetes Res. 2019;2019:2583047.

265 Chen J., Zhao H., Ma X.*, et al.* Glp-1/Glp-1r Signaling in Regulation of Adipocyte Differentiation and Lipogenesis. Cell Physiol Biochem. 2017;42(3):1165-76.

266 Hallbrink M., Holmqvist T., Olsson M.*, et al.* Different Domains in the Third Intracellular Loop of the Glp-1 Receptor Are Responsible for Galpha(S) and Galpha(I)/Galpha(O) Activation. Biochim Biophys Acta. 2001;1546(1):79-86.

267 Montrose-Rafizadeh C., Avdonin P., Garant M. J.*, et al.* Pancreatic Glucagon-Like Peptide-1 Receptor Couples to Multiple G Proteins and Activates Mitogen-Activated Protein Kinase Pathways in Chinese Hamster Ovary Cells. Endocrinology. 1999;140(3):1132-40.

268 Shigeto M., Cha C. Y., Rorsman P.*, et al.* A Role of Plc/Pkc-Dependent Pathway in Glp-1-Stimulated Insulin Secretion. Journal of Molecular Medicine. 2017;95(4):361-8.

269 Thompson A., Kanamarlapudi V. Agonist-Induced Internalisation of the Glucagon-Like Peptide-1 Receptor Is Mediated by the Galphaq Pathway. Biochem Pharmacol. 2015;93(1):72-84.

270 Lohse M. J., Benovic J. L., Codina J.*, et al.* Beta-Arrestin: A Protein That Regulates Beta-Adrenergic Receptor Function. Science. 1990;248(4962):1547-50.

271 Beautrait A., Paradis J. S., Zimmerman B.*, et al.* A New Inhibitor of the B-Arrestin/Ap2 Endocytic Complex Reveals Interplay between Gpcr Internalization and Signalling. Nature Communications. 2017;8(1):15054.

272 Laporte S. A., Oakley R. H., Zhang J.*, et al.* The Beta2-Adrenergic Receptor/Betaarrestin Complex Recruits the Clathrin Adaptor Ap-2 During Endocytosis. Proc Natl Acad Sci U S A. 1999;96(7):3712-7.

273 Al-Sabah S., Al-Fulaij M., Shaaban G.*, et al.* The Gip Receptor Displays Higher Basal Activity Than the Glp-1 Receptor but Does Not Recruit Grk2 or Arrestin3 Effectively. PLoS One. 2014;9(9):e106890.

274 Jorgensen R., Martini L., Schwartz T. W.*, et al.* Characterization of Glucagon-Like Peptide-1 Receptor Beta-Arrestin 2 Interaction: A High-Affinity Receptor Phenotype. Mol Endocrinol. 2005;19(3):812-23.

275 Sonoda N., Imamura T., Yoshizaki T.*, et al.* Beta-Arrestin-1 Mediates Glucagon-Like Peptide-1 Signaling to Insulin Secretion in Cultured Pancreatic Beta Cells. Proc Natl Acad Sci U S A. 2008;105(18):6614-9.

276 Jones B., Buenaventura T., Kanda N.*, et al.* Targeting Glp-1 Receptor Trafficking to Improve Agonist Efficacy. Nature Communications. 2018;9(1):1602.

277 Iacovelli L., Felicioni M., Nisticò R.*, et al.* Selective Regulation of Recombinantly Expressed Mglu7 Metabotropic Glutamate Receptors by G Protein-Coupled Receptor Kinases and Arrestins. Neuropharmacology. 2014;77:303-12.

278 Luttrell L. M., Roudabush F. L., Choy E. W.*, et al.* Activation and Targeting of Extracellular Signal-Regulated Kinases by Beta-Arrestin Scaffolds. Proc Natl Acad Sci U S A. 2001;98(5):2449-54.

279 Fletcher M. M., Halls M. L., Zhao P.*, et al.* Glucagon-Like Peptide-1 Receptor Internalisation Controls Spatiotemporal Signalling Mediated by Biased Agonists. Biochem Pharmacol. 2018;156:406-19.

280 Quoyer J., Longuet C., Broca C.*, et al.* Glp-1 Mediates Antiapoptotic Effect by Phosphorylating Bad through a Beta-Arrestin 1-Mediated Erk1/2 Activation in Pancreatic Beta-Cells. J Biol Chem. 2010;285(3):1989-2002.

281 Barella L. F., Rossi M., Zhu L.*, et al.* B-Cell-Intrinsic B-Arrestin 1 Signaling Enhances Sulfonylurea-Induced Insulin Secretion. J Clin Invest. 2019;130(9):3732-7.

282 Luan B., Zhao J., Wu H.*, et al.* Deficiency of a Beta-Arrestin-2 Signal Complex Contributes to Insulin Resistance. Nature. 2009;457(7233):1146-9.

283 Zhu L., Rossi M., Cui Y.*, et al.* Hepatic B-Arrestin 2 Is Essential for Maintaining Euglycemia. J Clin Invest. 2017;127(8):2941-5.

284 Zhuang L. N., Hu W. X., Zhang M. L.*, et al.* Beta-Arrestin-1 Protein Represses Diet-Induced Obesity. J Biol Chem. 2011;286(32):28396-402.

285 Ahrén B. Emerging Dipeptidyl Peptidase-4 Inhibitors for the Treatment of Diabetes. Expert Opin Emerg Drugs. 2008;13(4):593-607.

286 Aschner P., Kipnes M. S., Lunceford J. K.*, et al.* Effect of the Dipeptidyl Peptidase-4 Inhibitor Sitagliptin as Monotherapy on Glycemic Control in Patients with Type 2 Diabetes. Diabetes Care. 2006;29(12):2632-7.

287 Balas B., Baig M. R., Watson C.*, et al.* The Dipeptidyl Peptidase Iv Inhibitor Vildagliptin Suppresses Endogenous Glucose Production and Enhances Islet Function after Single-Dose Administration in Type 2 Diabetic Patients. J Clin Endocrinol Metab. 2007;92(4):1249-55.

288 Fadini G. P., Bottigliengo D., D'Angelo F.*, et al.* Comparative Effectiveness of Dpp-4 Inhibitors Versus Sulfonylurea for the Treatment of Type 2 Diabetes in Routine Clinical Practice: A Retrospective Multicenter Real-World Study. Diabetes Ther. 2018;9(4):1477-90.

289 Ahrén B. Novel Combination Treatment of Type 2 Diabetes Dpp-4 Inhibition + Metformin. Vasc Health Risk Manag. 2008;4(2):383-94.

290 Rosenstock J., Brazg R., Andryuk P. J.*, et al.* Efficacy and Safety of the Dipeptidyl Peptidase-4 Inhibitor Sitagliptin Added to Ongoing Pioglitazone Therapy in Patients with Type 2 Diabetes: A 24-Week, Multicenter, Randomized, Double-Blind, Placebo-Controlled, Parallel-Group Study. Clin Ther. 2006;28(10):1556-68.

291 Foley J. E., Jordan J. Weight Neutrality with the Dpp-4 Inhibitor, Vildagliptin: Mechanistic Basis and Clinical Experience. Vasc Health Risk Manag. 2010;6:541-8.

292 Brown D. X., Evans M. Choosing between Glp-1 Receptor Agonists and Dpp-4 Inhibitors: A Pharmacological Perspective. J Nutr Metab. 2012;2012:381713.

293 Pratley R., Nauck M., Bailey T.*, et al.* One Year of Liraglutide Treatment Offers Sustained and More Effective Glycaemic Control and Weight Reduction Compared with Sitagliptin, Both in Combination with Metformin, in Patients with Type 2 Diabetes: A Randomised, Parallel-Group, Open-Label Trial. Int J Clin Pract. 2011;65(4):397-407.

294 Pratley R. E., Nauck M., Bailey T.*, et al.* Liraglutide Versus Sitagliptin for Patients with Type 2 Diabetes Who Did Not Have Adequate Glycaemic Control with Metformin: A 26-Week, Randomised, Parallel-Group, Open-Label Trial. Lancet. 2010;375(9724):1447-56.

295 Brunton S. Glp-1 Receptor Agonists Vs. Dpp-4 Inhibitors for Type 2 Diabetes: Is One Approach More Successful or Preferable Than the Other? Int J Clin Pract. 2014;68(5):557-67.

296 Eng J., Kleinman W. A., Singh L.*, et al.* Isolation and Characterization of Exendin-4, an Exendin-3 Analogue, from Heloderma Suspectum Venom. Further Evidence for an Exendin Receptor on Dispersed Acini from Guinea Pig Pancreas. J Biol Chem. 1992;267(11):7402-5.

297 Schepp W., Schmidtler J., Riedel T.*, et al.* Exendin-4 and Exendin-(9-39)Nh2: Agonist and Antagonist, Respectively, at the Rat Parietal Cell Receptor for Glucagon-Like Peptide-1-(7-36)Nh2. Eur J Pharmacol. 1994;269(2):183-91.

298 Day J. W., Gelfanov V., Smiley D.*, et al.* Optimization of Co-Agonism at Glp-1 and Glucagon Receptors to Safely Maximize Weight Reduction in Dio-Rodents. Biopolymers. 2012;98(5):443-50.

299 Edwards C. M., Stanley S. A., Davis R.*, et al.* Exendin-4 Reduces Fasting and Postprandial Glucose and Decreases Energy Intake in Healthy Volunteers. Am J Physiol Endocrinol Metab. 2001;281(1):E155-61.

300 Gupta V. Glucagon-Like Peptide-1 Analogues: An Overview. Indian J Endocrinol Metab. 2013;17(3):413-21.

301 DeFronzo R. A., Ratner R. E., Han J.*, et al.* Effects of Exenatide (Exendin-4) on Glycemic Control and Weight over 30 Weeks in Metformin-Treated Patients with Type 2 Diabetes. Diabetes Care. 2005;28(5):1092-100.

302 Buse J. B., Bergenstal R. M., Glass L. C.*, et al.* Use of Twice-Daily Exenatide in Basal Insulin-Treated Patients with Type 2 Diabetes: A Randomized, Controlled Trial. Ann Intern Med. 2011;154(2):103-12.

303 Buse J. B., Henry R. R., Han J.*, et al.* Effects of Exenatide (Exendin-4) on Glycemic Control over 30 Weeks in Sulfonylurea-Treated Patients with Type 2 Diabetes. Diabetes Care. 2004;27(11):2628-35.

304 McCormack P. L. Exenatide Twice Daily: A Review of Its Use in the Management of Patients with Type 2 Diabetes Mellitus. Drugs. 2014;74(3):325-51.
305 Blevins T., Pullman J., Malloy J.*, et al.* Duration-5: Exenatide Once Weekly Resulted in Greater Improvements in Glycemic Control Compared with Exenatide Twice Daily in Patients with Type 2 Diabetes. J Clin Endocrinol Metab. 2011;96(5):1301-10.
306 Buse J. B., Drucker D. J., Taylor K. L.*, et al.* Duration-1: Exenatide Once Weekly Produces Sustained Glycemic Control and Weight Loss over 52 Weeks. Diabetes Care. 2010;33(6):1255-61.
307 Buse J. B., Nauck M., Forst T.*, et al.* Exenatide Once Weekly Versus Liraglutide Once Daily in Patients with Type 2 Diabetes (Duration-6): A Randomised, Open-Label Study. Lancet. 2013;381(9861):117-24.
308 Ahmann A. J., Capehorn M., Charpentier G.*, et al.* Efficacy and Safety of Once-Weekly Semaglutide Versus Exenatide Er in Subjects with Type 2 Diabetes (Sustain 3): A 56-Week, Open-Label, Randomized Clinical Trial. Diabetes Care. 2018;41(2):258-66.
309 Dalsgaard N. B., Vilsbøll T., Knop F. K. Effects of Glucagon-Like Peptide-1 Receptor Agonists on Cardiovascular Risk Factors: A Narrative Review of Head-to-Head Comparisons. Diabetes Obes Metab. 2018;20(3):508-19.
310 Nauck M. A., Petrie J. R., Sesti G.*, et al.* A Phase 2, Randomized, Dose-Finding Study of the Novel Once-Weekly Human Glp-1 Analog, Semaglutide, Compared with Placebo and Open-Label Liraglutide in Patients with Type 2 Diabetes. Diabetes Care. 2016;39(2):231-41.
311 Nuhoho S., Gupta J., Hansen B. B.*, et al.* Orally Administered Semaglutide Versus Glp-1 Ras in Patients with Type 2 Diabetes Previously Receiving 1-2 Oral Antidiabetics: Systematic Review and Network Meta-Analysis. Diabetes Ther. 2019;10(6):2183-99.
312 Pratley R. E., Aroda V. R., Lingvay I.*, et al.* Semaglutide Versus Dulaglutide Once Weekly in Patients with Type 2 Diabetes (Sustain 7): A Randomised, Open-Label, Phase 3b Trial. Lancet Diabetes Endocrinol. 2018;6(4):275-86.
313 Derosa G., Franzetti I. G., Querci F.*, et al.* Exenatide Plus Metformin Compared with Metformin Alone on B-Cell Function in Patients with Type 2 Diabetes. Diabet Med. 2012;29(12):1515-23.
314 Drucker D. J., Buse J. B., Taylor K.*, et al.* Exenatide Once Weekly Versus Twice Daily for the Treatment of Type 2 Diabetes: A Randomised, Open-Label, Non-Inferiority Study. Lancet. 2008;372(9645):1240-50.
315 Dushay J., Gao C., Gopalakrishnan G. S.*, et al.* Short-Term Exenatide Treatment Leads to Significant Weight Loss in a Subset of Obese Women without Diabetes. Diabetes Care. 2012;35(1):4-11.
316 Guja C., Frías J. P., Somogyi A.*, et al.* Effect of Exenatide Qw or Placebo, Both Added to Titrated Insulin Glargine, in Uncontrolled Type 2 Diabetes: The Duration-7 Randomized Study. Diabetes Obes Metab. 2018;20(7):1602-14.
317 Astrup A., Carraro R., Finer N.*, et al.* Safety, Tolerability and Sustained Weight Loss over 2 Years with the Once-Daily Human Glp-1 Analog, Liraglutide. Int J Obes (Lond). 2012;36(6):843-54.
318 Astrup A., Rössner S., Van Gaal L.*, et al.* Effects of Liraglutide in the Treatment of Obesity: A Randomised, Double-Blind, Placebo-Controlled Study. Lancet. 2009;374(9701):1606-16.

319 Blackman A., Foster G. D., Zammit G.*, et al.* Effect of Liraglutide 3.0 Mg in Individuals with Obesity and Moderate or Severe Obstructive Sleep Apnea: The Scale Sleep Apnea Randomized Clinical Trial. Int J Obes (Lond). 2016;40(8):1310-9.

320 Davies M. J., Bergenstal R., Bode B.*, et al.* Efficacy of Liraglutide for Weight Loss among Patients with Type 2 Diabetes: The Scale Diabetes Randomized Clinical Trial. Jama. 2015;314(7):687-99.

321 Pi-Sunyer X., Astrup A., Fujioka K.*, et al.* A Randomized, Controlled Trial of 3.0 Mg of Liraglutide in Weight Management. N Engl J Med. 2015;373(1):11-22.

322 Wadden T. A., Hollander P., Klein S.*, et al.* Weight Maintenance and Additional Weight Loss with Liraglutide after Low-Calorie-Diet-Induced Weight Loss: The Scale Maintenance Randomized Study. Int J Obes (Lond). 2013;37(11):1443-51.

323 Ahrén B., Atkin S. L., Charpentier G.*, et al.* Semaglutide Induces Weight Loss in Subjects with Type 2 Diabetes Regardless of Baseline Bmi or Gastrointestinal Adverse Events in the Sustain 1 to 5 Trials. Diabetes Obes Metab. 2018;20(9):2210-9.

324 O'Neil P. M., Birkenfeld A. L., McGowan B.*, et al.* Efficacy and Safety of Semaglutide Compared with Liraglutide and Placebo for Weight Loss in Patients with Obesity: A Randomised, Double-Blind, Placebo and Active Controlled, Dose-Ranging, Phase 2 Trial. Lancet. 2018;392(10148):637-49.

325 Pratley R., Amod A., Hoff S. T.*, et al.* Oral Semaglutide Versus Subcutaneous Liraglutide and Placebo in Type 2 Diabetes (Pioneer 4): A Randomised, Double-Blind, Phase 3a Trial. Lancet. 2019;394(10192):39-50.

326 Bettge K., Kahle M., Abd El Aziz M. S.*, et al.* Occurrence of Nausea, Vomiting and Diarrhoea Reported as Adverse Events in Clinical Trials Studying Glucagon-Like Peptide-1 Receptor Agonists: A Systematic Analysis of Published Clinical Trials. Diabetes Obes Metab. 2017;19(3):336-47.

327 Pratley R. E., Nauck M. A., Barnett A. H.*, et al.* Once-Weekly Albiglutide Versus Once-Daily Liraglutide in Patients with Type 2 Diabetes Inadequately Controlled on Oral Drugs (Harmony 7): A Randomised, Open-Label, Multicentre, Non-Inferiority Phase 3 Study. Lancet Diabetes Endocrinol. 2014;2(4):289-97.

328 Secher A., Jelsing J., Baquero A. F.*, et al.* The Arcuate Nucleus Mediates Glp-1 Receptor Agonist Liraglutide-Dependent Weight Loss. J Clin Invest. 2014;124(10):4473-88.

329 Baggio L. L., Drucker D. J. Glucagon-Like Peptide-1 Receptors in the Brain: Controlling Food Intake and Body Weight. J Clin Invest. 2014;124(10):4223-6.

330 Leiter L. A., Mallory J. M., Wilson T. H.*, et al.* Gastrointestinal Safety across the Albiglutide Development Programme. Diabetes Obes Metab. 2016;18(9):930-5.

331 Lando H. M., Alattar M., Dua A. P. Elevated Amylase and Lipase Levels in Patients Using Glucagonlike Peptide-1 Receptor Agonists or Dipeptidyl-Peptidase-4 Inhibitors in the Outpatient Setting. Endocr Pract. 2012;18(4):472-7.

332 Meier J. J., Nauck M. A. Risk of Pancreatitis in Patients Treated with Incretin-Based Therapies. Diabetologia. 2014;57(7):1320-4.

333 Singh S., Chang H. Y., Richards T. M.*, et al.* Glucagonlike Peptide 1-Based Therapies and Risk of Hospitalization for Acute Pancreatitis in Type 2 Diabetes Mellitus: A Population-Based Matched Case-Control Study. JAMA Intern Med. 2013;173(7):534-9.

334 Alves C., Batel-Marques F., Macedo A. F. A Meta-Analysis of Serious Adverse Events Reported with Exenatide and Liraglutide: Acute Pancreatitis and Cancer. Diabetes Res Clin Pract. 2012;98(2):271-84.
335 Funch D., Gydesen H., Tornøe K.*, et al.* A Prospective, Claims-Based Assessment of the Risk of Pancreatitis and Pancreatic Cancer with Liraglutide Compared to Other Antidiabetic Drugs. Diabetes Obes Metab. 2014;16(3):273-5.
336 Li L., Shen J., Bala M. M.*, et al.* Incretin Treatment and Risk of Pancreatitis in Patients with Type 2 Diabetes Mellitus: Systematic Review and Meta-Analysis of Randomised and Non-Randomised Studies. Bmj. 2014;348:g2366.
337 Yang L., He Z., Tang X.*, et al.* Type 2 Diabetes Mellitus and the Risk of Acute Pancreatitis: A Meta-Analysis. Eur J Gastroenterol Hepatol. 2013;25(2):225-31.
338 Scheen A. Gliptins (Dipeptidyl Peptidase-4 Inhibitors) and Risk of Acute Pancreatitis. Expert Opin Drug Saf. 2013;12(4):545-57.
339 Pontiroli A. E., Calderara A., Perfetti M. G.*, et al.* Pharmacokinetics of Intranasal, Intramuscular and Intravenous Glucagon in Healthy Subjects and Diabetic Patients. European Journal of Clinical Pharmacology. 1993;45(6):555-8.
340 Sandoval D. A., D'Alessio D. A. Physiology of Proglucagon Peptides: Role of Glucagon and Glp-1 in Health and Disease. Physiol Rev. 2015;95(2):513-48.
341 Briant L., Salehi A., Vergari E.*, et al.* Glucagon Secretion from Pancreatic A-Cells. Ups J Med Sci. 2016;121(2):113-9.
342 Thorens B. Brain Glucose Sensing and Neural Regulation of Insulin and Glucagon Secretion. Diabetes Obes Metab. 2011;13 Suppl 1:82-8.
343 Zhou C., Teegala S. B., Khan B. A.*, et al.* Hypoglycemia: Role of Hypothalamic Glucose-Inhibited (Gi) Neurons in Detection and Correction. Front Physiol. 2018;9:192.
344 Katsura T., Kawamori D., Aida E.*, et al.* Glucotoxicity Induces Abnormal Glucagon Secretion through Impaired Insulin Signaling in Inr1g Cells. PLoS One. 2017;12(4):e0176271.
345 Svoboda M., Tastenoy M., Vertongen P.*, et al.* Relative Quantitative Analysis of Glucagon Receptor Mrna in Rat Tissues. Mol Cell Endocrinol. 1994;105(2):131-7.
346 Miller R. A., Birnbaum M. J. Glucagon: Acute Actions on Hepatic Metabolism. Diabetologia. 2016;59(7):1376-81.
347 Rui L. Energy Metabolism in the Liver. Compr Physiol. 2014;4(1):177-97.
348 Unger R. H., Eisentraut A. M., Mc C. M.*, et al.* Glucagon Antibodies and an Immunoassay for Glucagon. J Clin Invest. 1961;40(7):1280-9.
349 Unger R. H., Orci L. The Essential Role of Glucagon in the Pathogenesis of Diabetes Mellitus. Lancet. 1975;1(7897):14-6.
350 Holst J. J., Wewer Albrechtsen N. J., Pedersen J.*, et al.* Glucagon and Amino Acids Are Linked in a Mutual Feedback Cycle: The Liver-A-Cell Axis. Diabetes. 2017;66(2):235-40.
351 Mallinson C. N., Bloom S. R., Warin A. P.*, et al.* A Glucagonoma Syndrome. Lancet. 1974;2(7871):1-5.
352 Thiessen S. E., Gunst J., Van den Berghe G. Role of Glucagon in Protein Catabolism. Curr Opin Crit Care. 2018;24(4):228-34.
353 Brand C. L., Rolin B., Jørgensen P. N.*, et al.* Immunoneutralization of Endogenous Glucagon with Monoclonal Glucagon Antibody Normalizes Hyperglycaemia in Moderately Streptozotocin-Diabetic Rats. Diabetologia. 1994;37(10):985-93.

354 Gelling R. W., Du X. Q., Dichmann D. S.*, et al.* Lower Blood Glucose, Hyperglucagonemia, and Pancreatic Alpha Cell Hyperplasia in Glucagon Receptor Knockout Mice. Proc Natl Acad Sci U S A. 2003;100(3):1438-43.

355 Holst J. J., Madsen O. G., Knop J.*, et al.* The Effect of Intraportal and Peripheral Infusions of Glucagon on Insulin and Glucose Concentrations and Glucose Tolerance in Normal Man. Diabetologia. 1977;13(5):487-90.

356 Sherwin R. S., Fisher M., Hendler R.*, et al.* Hyperglucagonemia and Blood Glucose Regulation in Normal, Obese and Diabetic Subjects. N Engl J Med. 1976;294(9):455-61.

357 Bloom S. R., Polak J. M. Glucagonoma Syndrome. Am J Med. 1987;82(5b):25-36.

358 Boden G., Rezvani I., Owen O. E. Effects of Glucagon on Plasma Amino Acids. J Clin Invest. 1984;73(3):785-93.

359 Charlton M. R., Adey D. B., Nair K. S. Evidence for a Catabolic Role of Glucagon During an Amino Acid Load. J Clin Invest. 1996;98(1):90-9.

360 Galsgaard K. D., Winther-Sorensen M., Orskov C.*, et al.* Disruption of Glucagon Receptor Signaling Causes Hyperaminoacidemia Exposing a Possible Liver-Alpha-Cell Axis. Am J Physiol Endocrinol Metab. 2018;314(1):E93-e103.

361 Almdal T. P., Holst J. J., Heindorff H.*, et al.* Glucagon Immunoneutralization in Diabetic Rats Normalizes Urea Synthesis and Decreases Nitrogen Wasting. Diabetes. 1992;41(1):12-6.

362 Hamberg O., Vilstrup H. Regulation of Urea Synthesis by Glucose and Glucagon in Normal Man. Clin Nutr. 1994;13(3):183-91.

363 Galsgaard K. D., Pedersen J., Knop F. K.*, et al.* Glucagon Receptor Signaling and Lipid Metabolism. Front Physiol. 2019;10:413.

364 Goodridge A. G. Regulation of the Gene for Fatty Acid Synthase. Fed Proc. 1986;45(9):2399-405.

365 Pégorier J. P., Garcia-Garcia M. V., Prip-Buus C.*, et al.* Induction of Ketogenesis and Fatty Acid Oxidation by Glucagon and Cyclic Amp in Cultured Hepatocytes from Rabbit Fetuses. Evidence for a Decreased Sensitivity of Carnitine Palmitoyltransferase I to Malonyl-Coa Inhibition after Glucagon or Cyclic Amp Treatment. Biochem J. 1989;264(1):93-100.

366 Jensen M. D., Heiling V. J., Miles J. M. Effects of Glucagon on Free Fatty Acid Metabolism in Humans. J Clin Endocrinol Metab. 1991;72(2):308-15.

367 Wu M. S., Jeng C. Y., Hollenbeck C. B.*, et al.* Does Glucagon Increase Plasma Free Fatty Acid Concentration in Humans with Normal Glucose Tolerance? J Clin Endocrinol Metab. 1990;70(2):410-6.

368 Xiao C., Pavlic M., Szeto L.*, et al.* Effects of Acute Hyperglucagonemia on Hepatic and Intestinal Lipoprotein Production and Clearance in Healthy Humans. Diabetes. 2011;60(2):383-90.

369 Samols E., Marri G., Marks V. Promotion of Insulin Secretion by Glucagon. Lancet. 1965;2(7409):415-6.

370 Goldfine I. D., Cerasi E., Luft R. Glucagon Stimulation of Insulin Release in Man: Inhibition During Hypoglycemia. J Clin Endocrinol Metab. 1972;35(2):312-5.

371 Liljenquist J. E., Bomboy J. D., Lewis S. B.*, et al.* Effects of Glucagon on Lipolysis and Ketogenesis in Normal and Diabetic Men. J Clin Invest. 1974;53(1):190-7.

372 Schneider S. H., Fineberg S. E., Blackburn G. L. The Acute Metabolic Effects of Glucagon and Its Interactions with Insulin in Forearm Tissue. Diabetologia. 1981;20(6):616-21.
373 Penick S. B., Hinkle L. E., Jr. Depression of Food Intake Induced in Healthy Subjects by Glucagon. N Engl J Med. 1961;264:893-7.
374 Schulman J. L., Carleton J. L., Whitney G.*, et al.* Effect of Glucagon on Food Intake and Body Weight in Man. J Appl Physiol. 1957;11(3):419-21.
375 Stunkard A. J., Van Itallie T. B., Reis B. B. The Mechanism of Satiety: Effect of Glucagon on Gastric Hunger Contractions in Man. Proc Soc Exp Biol Med. 1955;89(2):258-61.
376 Bagger J. I., Holst J. J., Hartmann B.*, et al.* Effect of Oxyntomodulin, Glucagon, Glp-1, and Combined Glucagon +Glp-1 Infusion on Food Intake, Appetite, and Resting Energy Expenditure. J Clin Endocrinol Metab. 2015;100(12):4541-52.
377 Banks W. A., Kastin A. J. Peptides and the Blood-Brain Barrier: Lipophilicity as a Predictor of Permeability. Brain Res Bull. 1985;15(3):287-92.
378 Geary N., Smith G. P. Selective Hepatic Vagotomy Blocks Pancreatic Glucagon's Satiety Effect. Physiol Behav. 1983;31(3):391-4.
379 Geary N., Kissileff H. R., Pi-Sunyer F. X.*, et al.* Individual, but Not Simultaneous, Glucagon and Cholecystokinin Infusions Inhibit Feeding in Men. Am J Physiol. 1992;262(6 Pt 2):R975-80.
380 SALTER J. M. Metabolic Effects of Glucagon in the Wistar Rat. The American Journal of Clinical Nutrition. 1960;8(5):535-9.
381 Salem V., Izzi-Engbeaya C., Coello C.*, et al.* Glucagon Increases Energy Expenditure Independently of Brown Adipose Tissue Activation in Humans. Diabetes Obes Metab. 2016;18(1):72-81.
382 Salter J. M., Ezrin C., Laidlaw J. C.*, et al.* Metabolic Effects of Glucagon in Human Subjects. Metabolism: clinical and experimental. 1960;9:753-68.
383 Billington C. J., Briggs J. E., Link J. G.*, et al.* Glucagon in Physiological Concentrations Stimulates Brown Fat Thermogenesis in Vivo. Am J Physiol. 1991;261(2 Pt 2):R501-7.
384 Doi K., Kuroshima A. Modified Metabolic Responsiveness to Glucagon in Cold-Acclimated and Heat-Acclimated Rats. Life Sci. 1982;30(9):785-91.
385 Kinoshita K., Ozaki N., Takagi Y.*, et al.* Glucagon Is Essential for Adaptive Thermogenesis in Brown Adipose Tissue. Endocrinology. 2014;155(9):3484-92.
386 Seitz H. J., Krone W., Wilke H.*, et al.* Rapid Rise in Plasma Glucagon Induced by Acute Cold Exposure in Man and Rat. Pflugers Arch. 1981;389(2):115-20.
387 Dicker A., Zhao J., Cannon B.*, et al.* Apparent Thermogenic Effect of Injected Glucagon Is Not Due to a Direct Effect on Brown Fat Cells. Am J Physiol. 1998;275(5):R1674-82.
388 Townsend L. K., Medak K. D., Knuth C. M.*, et al.* Loss of Glucagon Signaling Alters White Adipose Tissue Browning. Faseb j. 2019;33(4):4824-35.
389 Carr R. D., Larsen M. O., Jelic K.*, et al.* Secretion and Dipeptidyl Peptidase-4-Mediated Metabolism of Incretin Hormones after a Mixed Meal or Glucose Ingestion in Obese Compared to Lean, Nondiabetic Men. J Clin Endocrinol Metab. 2010;95(2):872-8.
390 Huypens P., Ling Z., Pipeleers D.*, et al.* Glucagon Receptors on Human Islet Cells Contribute to Glucose Competence of Insulin Release. Diabetologia. 2000;43(8):1012-9.

391 Capozzi M. E., Wait J. B., Koech J.*, et al.* Glucagon Lowers Glycemia When B-Cells Are Active. JCI Insight. 2019;5(16).
392 Zhu L., Dattaroy D., Pham J.*, et al.* Intra-Islet Glucagon Signaling Is Critical for Maintaining Glucose Homeostasis. JCI Insight. 2019;5(10).
393 Gelling R. W., Vuguin P. M., Du X. Q.*, et al.* Pancreatic Beta-Cell Overexpression of the Glucagon Receptor Gene Results in Enhanced Beta-Cell Function and Mass. Am J Physiol Endocrinol Metab. 2009;297(3):E695-707.
394 Kim T., Holleman C. L., Nason S.*, et al.* Hepatic Glucagon Receptor Signaling Enhances Insulin-Stimulated Glucose Disposal in Rodents. Diabetes. 2018;67(11):2157-66.
395 Altarejos J. Y., Montminy M. Creb and the Crtc Co-Activators: Sensors for Hormonal and Metabolic Signals. Nat Rev Mol Cell Biol. 2011;12(3):141-51.
396 Quinn P. G., Granner D. K. Cyclic Amp-Dependent Protein Kinase Regulates Transcription of the Phosphoenolpyruvate Carboxykinase Gene but Not Binding of Nuclear Factors to the Cyclic Amp Regulatory Element. Mol Cell Biol. 1990;10(7):3357-64.
397 Longuet C., Sinclair E., Maida A.*, et al.* The Glucagon Receptor Is Required for the Adaptive Metabolic Response to Fasting. Cell Metabolism. 2008;8:359-71.
398 Aromataris E. C., Roberts M. L., Barritt G. J.*, et al.* Glucagon Activates Ca2+ and Cl- Channels in Rat Hepatocytes. J Physiol. 2006;573(Pt 3):611-25.
399 Wakelam M. J., Murphy G. J., Hruby V. J.*, et al.* Activation of Two Signal-Transduction Systems in Hepatocytes by Glucagon. Nature. 1986;323(6083):68-71.
400 Xu Y., Xie X. Glucagon Receptor Mediates Calcium Signaling by Coupling to Gαq/11 and Gαi/O in Hek293 Cells. Journal of Receptors and Signal Transduction. 2009;29(6):318-25.
401 Screaton R. A., Conkright M. D., Katoh Y.*, et al.* The Creb Coactivator Torc2 Functions as a Calcium- and Camp-Sensitive Coincidence Detector. Cell. 2004;119(1):61-74.
402 Ikegami T., Krilov L., Meng J.*, et al.* Decreased Glucagon Responsiveness by Bile Acids: A Role for Protein Kinase Calpha and Glucagon Receptor Phosphorylation. Endocrinology. 2006;147(11):5294-302.
403 Krilov L., Nguyen A., Miyazaki T.*, et al.* Dual Mode of Glucagon Receptor Internalization: Role of Pkcalpha, Grks and Beta-Arrestins. Exp Cell Res. 2011;317(20):2981-94.
404 McShane L. M., Irwin N., O'Flynn D.*, et al.* Glucagon Receptor Antagonist and Gip Agonist Combination for Diet-Induced Obese Mice. J Endocrinol. 2016;229(3):319-30.
405 Okamoto H., Cavino K., Na E.*, et al.* Glucagon Receptor Inhibition Normalizes Blood Glucose in Severe Insulin-Resistant Mice. Proc Natl Acad Sci U S A. 2017;114(10):2753-8.
406 Qureshi S. A., Rios Candelore M., Xie D.*, et al.* A Novel Glucagon Receptor Antagonist Inhibits Glucagon-Mediated Biological Effects. Diabetes. 2004;53(12):3267-73.
407 Pearson M. J., Unger R. H., Holland W. L. Clinical Trials, Triumphs, and Tribulations of Glucagon Receptor Antagonists. Diabetes Care. 2016;39(7):1075-7.
408 Guan H. P., Yang X., Lu K.*, et al.* Glucagon Receptor Antagonism Induces Increased Cholesterol Absorption. J Lipid Res. 2015;56(11):2183-95.
409 Gravholt C. H., Møller N., Jensen M. D.*, et al.* Physiological Levels of Glucagon Do Not Influence Lipolysis in Abdominal Adipose Tissue as Assessed by Microdialysis. J Clin Endocrinol Metab. 2001;86(5):2085-9.

410 Guettet C., Mathe D., Riottot M*., et al.* Effects of Chronic Glucagon Administration on Cholesterol and Bile Acid Metabolism. Biochim Biophys Acta. 1988;963(2):215-23.
411 Guettet C., Rostaqui N., Mathé D*., et al.* Effect of Chronic Glucagon Administration on Lipoprotein Composition in Normally Fed, Fasted and Cholesterol-Fed Rats. Lipids. 1991;26(6):451-8.
412 Penhos J. C., Wu C. H., Daunas J*., et al.* Effect of Glucagon on the Metabolism of Lipids and on Urea Formation by the Perfused Rat Liver. Diabetes. 1966;15(10):740-8.
413 Moh Moh M. A., Jung C. H., Lee B*., et al.* Association of Glucagon-to-Insulin Ratio and Nonalcoholic Fatty Liver Disease in Patients with Type 2 Diabetes Mellitus. Diab Vasc Dis Res. 2019;16(2):186-95.
414 Suppli M. P., Bagger J. I., Lund A*., et al.* Glucagon Resistance at the Level of Amino Acid Turnover in Obese Subjects with Hepatic Steatosis. Diabetes. 2020;69(6):1090-9.
415 Suppli M. P., Lund A., Bagger J. I*., et al.* Involvement of Steatosis-Induced Glucagon Resistance in Hyperglucagonaemia. Med Hypotheses. 2016;86:100-3.
416 Finan B., Clemmensen C., Zhu Z*., et al.* Chemical Hybridization of Glucagon and Thyroid Hormone Optimizes Therapeutic Impact for Metabolic Disease. Cell. 2016;167(3):843-57.e14.
417 Mullur R., Liu Y. Y., Brent G. A. Thyroid Hormone Regulation of Metabolism. Physiol Rev. 2014;94(2):355-82.
418 Pucci E., Chiovato L., Pinchera A. Thyroid and Lipid Metabolism. Int J Obes Relat Metab Disord. 2000;24 Suppl 2:S109-12.
419 Dayan C., Panicker V. Management of Hypothyroidism with Combination Thyroxine (T4) and Triiodothyronine (T3) Hormone Replacement in Clinical Practice: A Review of Suggested Guidance. Thyroid Res. 2018;11:1.
420 Holst J. J. Enteroglucagon. Annu Rev Physiol. 1997;59:257-71.
421 Dakin C. L., Gunn I., Small C. J*., et al.* Oxyntomodulin Inhibits Food Intake in the Rat. Endocrinology. 2001;142(10):4244-50.
422 Jorgensen R., Kubale V., Vrecl M*., et al.* Oxyntomodulin Differentially Affects Glucagon-Like Peptide-1 Receptor Beta-Arrestin Recruitment and Signaling through Galpha(S). J Pharmacol Exp Ther. 2007;322(1):148-54.
423 Schjoldager B. T., Baldissera F. G., Mortensen P. E*., et al.* Oxyntomodulin: A Potential Hormone from the Distal Gut. Pharmacokinetics and Effects on Gastric Acid and Insulin Secretion in Man. Eur J Clin Invest. 1988;18(5):499-503.
424 Scott R., Minnion J., Tan T*., et al.* Oxyntomodulin Analogue Increases Energy Expenditure Via the Glucagon Receptor. Peptides. 2018;104:70-7.
425 Du X., Kosinski J. R., Lao J*., et al.* Differential Effects of Oxyntomodulin and Glp-1 on Glucose Metabolism. Am J Physiol Endocrinol Metab. 2012;303(2):E265-71.
426 Field B. C., Wren A. M., Peters V*., et al.* Pyy3-36 and Oxyntomodulin Can Be Additive in Their Effect on Food Intake in Overweight and Obese Humans. Diabetes. 2010;59(7):1635-9.
427 Shankar S. S., Shankar R. R., Mixson L. A*., et al.* Native Oxyntomodulin Has Significant Glucoregulatory Effects Independent of Weight Loss in Obese Humans with and without Type 2 Diabetes. Diabetes. 2018;67(6):1105-12.
428 Cohen M. A., Ellis S. M., Le Roux C. W*., et al.* Oxyntomodulin Suppresses Appetite and Reduces Food Intake in Humans. J Clin Endocrinol Metab. 2003;88(10):4696-701.

429 Dakin C. L., Small C. J., Batterham R. L.*, et al.* Peripheral Oxyntomodulin Reduces Food Intake and Body Weight Gain in Rats. Endocrinology. 2004;145(6):2687-95.

430 Wynne K., Park A. J., Small C. J.*, et al.* Oxyntomodulin Increases Energy Expenditure in Addition to Decreasing Energy Intake in Overweight and Obese Humans: A Randomised Controlled Trial. Int J Obes (Lond). 2006;30(12):1729-36.

431 Wynne K., Park A. J., Small C. J.*, et al.* Subcutaneous Oxyntomodulin Reduces Body Weight in Overweight and Obese Subjects: A Double-Blind, Randomized, Controlled Trial. Diabetes. 2005;54(8):2390-5.

432 Chaudhri O. B., Parkinson J. R., Kuo Y. T.*, et al.* Differential Hypothalamic Neuronal Activation Following Peripheral Injection of Glp-1 and Oxyntomodulin in Mice Detected by Manganese-Enhanced Magnetic Resonance Imaging. Biochem Biophys Res Commun. 2006;350(2):298-306.

433 Parkinson J. R., Chaudhri O. B., Kuo Y. T.*, et al.* Differential Patterns of Neuronal Activation in the Brainstem and Hypothalamus Following Peripheral Injection of Glp-1, Oxyntomodulin and Lithium Chloride in Mice Detected by Manganese-Enhanced Magnetic Resonance Imaging (Memri). Neuroimage. 2009;44(3):1022-31.

434 Sowden G. L., Drucker D. J., Weinshenker D.*, et al.* Oxyntomodulin Increases Intrinsic Heart Rate in Mice Independent of the Glucagon-Like Peptide-1 Receptor. Am J Physiol Regul Integr Comp Physiol. 2007;292(2):R962-70.

435 Günther T., Dasgupta P., Mann A.*, et al.* Targeting Multiple Opioid Receptors - Improved Analgesics with Reduced Side Effects? Br J Pharmacol. 2018;175(14):2857-68.

436 Day J. W., Ottaway N., Patterson J. T.*, et al.* A New Glucagon and Glp-1 Co-Agonist Eliminates Obesity in Rodents. Nat Chem Biol. 2009;5(10):749-57.

437 Pocai A., Carrington P. E., Adams J. R.*, et al.* Glucagon-Like Peptide 1/Glucagon Receptor Dual Agonism Reverses Obesity in Mice. Diabetes. 2009;58(10):2258-66.

438 ClinicalTrials.gov. A Preliminary Study of the Efficacy and Safety of Mk-8521 for Type 2 Diabetes (Mk-8521-004) 2018 [Available from: https://clinicaltrials.gov/ct2/show/results/NCT02492763.

439 Tillner J., Posch M. G., Wagner F.*, et al.* A Novel Dual Glucagon-Like Peptide and Glucagon Receptor Agonist Sar425899: Results of Randomized, Placebo-Controlled First-in-Human and First-in-Patient Trials. Diabetes Obes Metab. 2019;21(1):120-8.

440 Goebel B., Schiavon M., Visentin R.*, et al.* Effects of the Novel Dual Glp-1r/Gcgr Agonist Sar425899 on Postprandial Glucose Metabolism in Overweight/Obese Subjects with Type 2 Diabetes. Diabetes. 2018;67(Supplement 1):72-OR.

441 Visentin R., Schiavon M., Göbel B.*, et al.* Dual Glucagon-Like Peptide-1 Receptor/Glucagon Receptor Agonist Sar425899 Improves Beta-Cell Function in Type 2 Diabetes. Diabetes Obes Metab. 2020;22(4):640-7.

442 Henderson S. J., Konkar A., Hornigold D. C.*, et al.* Robust Anti-Obesity and Metabolic Effects of a Dual Glp-1/Glucagon Receptor Peptide Agonist in Rodents and Non-Human Primates. Diabetes Obes Metab. 2016;18(12):1176-90.

443 Ambery P., Parker V. E., Stumvoll M.*, et al.* Medi0382, a Glp-1 and Glucagon Receptor Dual Agonist, in Obese or Overweight Patients with Type 2 Diabetes: A Randomised, Controlled, Double-Blind, Ascending Dose and Phase 2a Study. Lancet. 2018;391(10140):2607-18.

444 Jain M., Tsai L. F., Robertson D.*, et al.* Medi0382, a Glp/Glucagon Receptor Dual Agonist, Significantly Reduces Hepatic Fat Content in Subjects with Type 2 Diabetes Mellitus. American Diabetes Association. 2018;67.

445 Brandt S. J., Götz A., Tschöp M. H.*, et al.* Gut Hormone Polyagonists for the Treatment of Type 2 Diabetes. Peptides. 2018;100:190-201.

446 Evers A., Haack T., Lorenz M.*, et al.* Design of Novel Exendin-Based Dual Glucagon-Like Peptide 1 (Glp-1)/Glucagon Receptor Agonists. J Med Chem. 2017;60(10):4293-303.

447 Zhou J., Cai X., Huang X.*, et al.* A Novel Glucagon-Like Peptide-1/Glucagon Receptor Dual Agonist Exhibits Weight-Lowering and Diabetes-Protective Effects. Eur J Med Chem. 2017;138:1158-69.

448 Althage M. C., Ford E. L., Wang S.*, et al.* Targeted Ablation of Glucose-Dependent Insulinotropic Polypeptide-Producing Cells in Transgenic Mice Reduces Obesity and Insulin Resistance Induced by a High Fat Diet. J Biol Chem. 2008;283(26):18365-76.

449 Miyawaki K., Yamada Y., Yano H.*, et al.* Glucose Intolerance Caused by a Defect in the Entero-Insular Axis: A Study in Gastric Inhibitory Polypeptide Receptor Knockout Mice. Proc Natl Acad Sci U S A. 1999;96(26):14843-7.

450 Gault V. A., O'Harte F. P., Harriott P.*, et al.* Characterization of the Cellular and Metabolic Effects of a Novel Enzyme-Resistant Antagonist of Glucose-Dependent Insulinotropic Polypeptide. Biochem Biophys Res Commun. 2002;290(5):1420-6.

451 McClean P. L., Irwin N., Cassidy R. S.*, et al.* Gip Receptor Antagonism Reverses Obesity, Insulin Resistance, and Associated Metabolic Disturbances Induced in Mice by Prolonged Consumption of High-Fat Diet. Am J Physiol Endocrinol Metab. 2007;293(6):E1746-55.

452 Kim S. J., Nian C., Karunakaran S.*, et al.* Gip-Overexpressing Mice Demonstrate Reduced Diet-Induced Obesity and Steatosis, and Improved Glucose Homeostasis. PLoS One. 2012;7(7):e40156.

453 Hinke S. A., Gelling R. W., Pederson R. A.*, et al.* Dipeptidyl Peptidase Iv-Resistant [D-Ala(2)]Glucose-Dependent Insulinotropic Polypeptide (Gip) Improves Glucose Tolerance in Normal and Obese Diabetic Rats. Diabetes. 2002;51(3):652-61.

454 Widenmaier S. B., Kim S. J., Yang G. K.*, et al.* A Gip Receptor Agonist Exhibits Beta-Cell Anti-Apoptotic Actions in Rat Models of Diabetes Resulting in Improved Beta-Cell Function and Glycemic Control. PLoS One. 2010;5(3):e9590.

455 Coskun T., Sloop K. W., Loghin C.*, et al.* Ly3298176, a Novel Dual Gip and Glp-1 Receptor Agonist for the Treatment of Type 2 Diabetes Mellitus: From Discovery to Clinical Proof of Concept. Mol Metab. 2018;18:3-14.

456 Finan B., Ma T., Ottaway N.*, et al.* Unimolecular Dual Incretins Maximize Metabolic Benefits in Rodents, Monkeys, and Humans. Sci Transl Med. 2013;5(209):209ra151.

457 Gault V. A., Kerr B. D., Harriott P.*, et al.* Administration of an Acylated Glp-1 and Gip Preparation Provides Added Beneficial Glucose-Lowering and Insulinotropic Actions over Single Incretins in Mice with Type 2 Diabetes and Obesity. Clin Sci (Lond). 2011;121(3):107-17.

458 Schmitt C., Portron A., Jadidi S.*, et al.* Pharmacodynamics, Pharmacokinetics and Safety of Multiple Ascending Doses of the Novel Dual Glucose-Dependent Insulinotropic Polypeptide/Glucagon-Like Peptide-1 Agonist Rg7697 in People with Type 2 Diabetes Mellitus. Diabetes Obes Metab. 2017;19(10):1436-45.

459 Frias J. P., Bastyr E. J., 3rd, Vignati L.*, et al.* The Sustained Effects of a Dual Gip/Glp-1 Receptor Agonist, Nnc0090-2746, in Patients with Type 2 Diabetes. Cell Metab. 2017;26(2):343-52.e2.

460 Frias J. P., Nauck M. A., Van J.*, et al.* Efficacy and Tolerability of Tirzepatide, a Dual Glucose-Dependent Insulinotropic Peptide and Glucagon-Like Peptide-1 Receptor Agonist in Patients with Type 2 Diabetes: A 12-Week, Randomized, Double-Blind, Placebo-Controlled Study to Evaluate Different Dose-Escalation Regimens. Diabetes Obes Metab. 2020;22(6):938-46.

461 Frias J. P., Nauck M. A., Van J.*, et al.* Efficacy and Safety of Ly3298176, a Novel Dual Gip and Glp-1 Receptor Agonist, in Patients with Type 2 Diabetes: A Randomised, Placebo-Controlled and Active Comparator-Controlled Phase 2 Trial. Lancet. 2018;392(10160):2180-93.

462 Samms R. J., Christe M. E., Ruan X.*, et al.* 1009-P: The Dual Gip and Glp-1 Receptor Agonist Regulates Lipid and Carbohydrate Metabolism through Gipr in Adipose Tissue. American Diabetes Association. 2019;68.

463 Urva S., Nauck M., Coskun T.*, et al.* 58-Or: The Novel Dual Gip and Glp-1 Receptor Agonist Tirzepatide Transiently Delays Gastric Emptying Similarly to a Selective Long-Acting Glp-1 Receptor Agonist. American Diabetes Association. 2019;68.

464 Finan B., Yang B., Ottaway N.*, et al.* A Rationally Designed Monomeric Peptide Triagonist Corrects Obesity and Diabetes in Rodents. Nat Med. 2015;21(1):27-36.

465 Jall S., Sachs S., Clemmensen C.*, et al.* Monomeric Glp-1/Gip/Glucagon Triagonism Corrects Obesity, Hepatosteatosis, and Dyslipidemia in Female Mice. Mol Metab. 2017;6(5):440-6.

466 Choi I. Y., Kim J. K., Lee J. S.*, et al.* Effect of a Novel Long-Acting Glp-1/Gip/Glucagon Triple Agonist (Hm15211) in a Nash and Fibrosis Animal Model. American Diabetes Association. 2018;67:1106-P.

467 Leff P. The Two-State Model of Receptor Activation. Trends Pharmacol Sci. 1995;16(3):89-97.

468 Jarpe M. B., Knall C., Mitchell F. M.*, et al.* [D-Arg1,D-Phe5,D-Trp7,9,Leu11]Substance P Acts as a Biased Agonist toward Neuropeptide and Chemokine Receptors. J Biol Chem. 1998;273(5):3097-104.

469 Kenakin T. The Effective Application of Biased Signaling to New Drug Discovery. Mol Pharmacol. 2015;88(6):1055-61.

470 Wootten D., Christopoulos A., Marti-Solano M.*, et al.* Mechanisms of Signalling and Biased Agonism in G Protein-Coupled Receptors. Nat Rev Mol Cell Biol. 2018;19(10):638-53.

471 Liu R., Bu W., Xi J.*, et al.* Beyond the Detergent Effect: A Binding Site for Sodium Dodecyl Sulfate (Sds) in Mammalian Apoferritin. Acta Crystallogr D Biol Crystallogr. 2012;68(Pt 5):497-504.

472 Shimada I., Ueda T., Kofuku Y.*, et al.* Gpcr Drug Discovery: Integrating Solution Nmr Data with Crystal and Cryo-Em Structures. Nat Rev Drug Discov. 2019;18(1):59-82.

473 Ueda T., Kofuku Y., Okude J.*, et al.* Function-Related Conformational Dynamics of G Protein-Coupled Receptors Revealed by Nmr. Biophys Rev. 2019;11(3):409-18.

474 Smith J. S., Lefkowitz R. J., Rajagopal S. Biased Signalling: From Simple Switches to Allosteric Microprocessors. Nat Rev Drug Discov. 2018;17(4):243-60.

475 Strachan R. T., Sun J. P., Rominger D. H.*, et al.* Divergent Transducer-Specific Molecular Efficacies Generate Biased Agonism at a G Protein-Coupled Receptor (Gpcr). J Biol Chem. 2014;289(20):14211-24.
476 Kenakin T., Watson C., Muniz-Medina V.*, et al.* A Simple Method for Quantifying Functional Selectivity and Agonist Bias. ACS Chem Neurosci. 2012;3(3):193-203.
477 Ehlert F. J., Griffin M. T., Sawyer G. W.*, et al.* A Simple Method for Estimation of Agonist Activity at Receptor Subtypes: Comparison of Native and Cloned M3 Muscarinic Receptors in Guinea Pig Ileum and Transfected Cells. J Pharmacol Exp Ther. 1999;289(2):981-92.
478 Rajagopal S., Ahn S., Rominger D. H.*, et al.* Quantifying Ligand Bias at Seven-Transmembrane Receptors. Mol Pharmacol. 2011;80(3):367-77.
479 Stahl E. L., Zhou L., Ehlert F. J.*, et al.* A Novel Method for Analyzing Extremely Biased Agonism at G Protein-Coupled Receptors. Mol Pharmacol. 2015;87(5):866-77.
480 Gundry J., Glenn R., Alagesan P.*, et al.* A Practical Guide to Approaching Biased Agonism at G Protein Coupled Receptors. Front Neurosci. 2017;11:17.
481 Rajagopal S., Bassoni D. L., Campbell J. J.*, et al.* Biased Agonism as a Mechanism for Differential Signaling by Chemokine Receptors. J Biol Chem. 2013;288(49):35039-48.
482 Michel M. C., Charlton S. J. Biased Agonism in Drug Discovery-Is It Too Soon to Choose a Path? Mol Pharmacol. 2018;93(4):259-65.
483 Machelska H., Celik M. Advances in Achieving Opioid Analgesia without Side Effects. Front Pharmacol. 2018;9:1388.
484 Bohn L. M., Gainetdinov R. R., Caron M. G. G Protein-Coupled Receptor Kinase/Beta-Arrestin Systems and Drugs of Abuse: Psychostimulant and Opiate Studies in Knockout Mice. Neuromolecular Med. 2004;5(1):41-50.
485 Bohn L. M., Gainetdinov R. R., Lin F. T.*, et al.* Mu-Opioid Receptor Desensitization by Beta-Arrestin-2 Determines Morphine Tolerance but Not Dependence. Nature. 2000;408(6813):720-3.
486 Raehal K. M., Walker J. K., Bohn L. M. Morphine Side Effects in Beta-Arrestin 2 Knockout Mice. J Pharmacol Exp Ther. 2005;314(3):1195-201.
487 Przewlocka B., Sieja A., Starowicz K.*, et al.* Knockdown of Spinal Opioid Receptors by Antisense Targeting Beta-Arrestin Reduces Morphine Tolerance and Allodynia in Rat. Neurosci Lett. 2002;325(2):107-10.
488 Bohn L. M., Lefkowitz R. J., Gainetdinov R. R.*, et al.* Enhanced Morphine Analgesia in Mice Lacking Beta-Arrestin 2. Science. 1999;286(5449):2495-8.
489 Yang C.-H., Huang H.-W., Chen K.-H.*, et al.* Antinociceptive Potentiation and Attenuation of Tolerance by Intrathecal B-Arrestin 2 Small Interfering Rna in Rats†. BJA: British Journal of Anaesthesia. 2011;107(5):774-81.
490 DeWire S. M., Yamashita D. S., Rominger D. H.*, et al.* A G Protein-Biased Ligand at the M-Opioid Receptor Is Potently Analgesic with Reduced Gastrointestinal and Respiratory Dysfunction Compared with Morphine. J Pharmacol Exp Ther. 2013;344(3):708-17.
491 Soergel D. G., Subach R. A., Burnham N.*, et al.* Biased Agonism of the M-Opioid Receptor by Trv130 Increases Analgesia and Reduces on-Target Adverse Effects Versus Morphine: A Randomized, Double-Blind, Placebo-Controlled, Crossover Study in Healthy Volunteers. Pain. 2014;155(9):1829-35.
492 Viscusi E. R., Skobieranda F., Soergel D. G.*, et al.* Apollo-1: A Randomized Placebo and Active-Controlled Phase Iii Study Investigating Oliceridine (Trv130), a G Protein-

Biased Ligand at the M-Opioid Receptor, for Management of Moderate-to-Severe Acute Pain Following Bunionectomy. J Pain Res. 2019;12:927-43.

493 Viscusi E. R., Webster L., Kuss M.*, et al.* A Randomized, Phase 2 Study Investigating Trv130, a Biased Ligand of the M-Opioid Receptor, for the Intravenous Treatment of Acute Pain. Pain. 2016;157(1):264-72.

494 Manglik A., Lin H., Aryal D. K.*, et al.* Structure-Based Discovery of Opioid Analgesics with Reduced Side Effects. Nature. 2016;537(7619):185-90.

495 Frankowski K. J., Hedrick M. P., Gosalia P.*, et al.* Discovery of Small Molecule Kappa Opioid Receptor Agonist and Antagonist Chemotypes through a Hts and Hit Refinement Strategy. ACS Chem Neurosci. 2012;3(3):221-36.

496 Pradhan A. A., Perroy J., Walwyn W. M.*, et al.* Agonist-Specific Recruitment of Arrestin Isoforms Differentially Modify Delta Opioid Receptor Function. J Neurosci. 2016;36(12):3541-51.

497 Zhang B., Zhao S., Yang D.*, et al.* A Novel G Protein-Biased and Subtype-Selective Agonist for a G Protein-Coupled Receptor Discovered from Screening Herbal Extracts. ACS Cent Sci. 2020;6(2):213-25.

498 Jastrzębska-Więsek M., Partyka A., Rychtyk J.*, et al.* Activity of Serotonin 5-Ht(1a) Receptor Biased Agonists in Rat: Anxiolytic and Antidepressant-Like Properties. ACS Chem Neurosci. 2018;9(5):1040-50.

499 Violin J. D., DeWire S. M., Yamashita D.*, et al.* Selectively Engaging B-Arrestins at the Angiotensin Ii Type 1 Receptor Reduces Blood Pressure and Increases Cardiac Performance. J Pharmacol Exp Ther. 2010;335(3):572-9.

500 Smith J. S., Nicholson L. T., Suwanpradid J.*, et al.* Biased Agonists of the Chemokine Receptor Cxcr3 Differentially Control Chemotaxis and Inflammation. Sci Signal. 2018;11(555).

501 Randáková A., Nelic D., Ungerová D.*, et al.* Novel M(2) -Selective, G(I) -Biased Agonists of Muscarinic Acetylcholine Receptors. Br J Pharmacol. 2020;177(9):2073-89.

502 Zhang H., Sturchler E., Zhu J.*, et al.* Autocrine Selection of a Glp-1r G-Protein Biased Agonist with Potent Antidiabetic Effects. Nat Commun. 2015;6:8918.

503 Liang Y. L., Khoshouei M., Glukhova A.*, et al.* Phase-Plate Cryo-Em Structure of a Biased Agonist-Bound Human Glp-1 Receptor-Gs Complex. Nature. 2018;555(7694):121-5.

504 Wang M. Y., P.; Gao, M.; Jin, J.; Yu, Y. Novel Fatty Chain-Modified Glp-1r G Protein-Biased Agonist Exerts Prolonged Anti-Diabetic Effects through Targeting Receptor Binding Sites. RSC Advances. 2020;10(14):8044-53.

505 Hager M. V., Johnson L. M., Wootten D.*, et al.* B-Arrestin-Biased Agonists of the Glp-1 Receptor from B-Amino Acid Residue Incorporation into Glp-1 Analogues. J Am Chem Soc. 2016;138(45):14970-9.

506 Zhao P., Liang Y. L., Belousoff M. J.*, et al.* Activation of the Glp-1 Receptor by a Non-Peptidic Agonist. Nature. 2020;577(7790):432-6.

507 Koole C., Wootten D., Simms J.*, et al.* Second Extracellular Loop of Human Glucagon-Like Peptide-1 Receptor (Glp-1r) Has a Critical Role in Glp-1 Peptide Binding and Receptor Activation. J Biol Chem. 2012;287(6):3642-58.

508 Yin Y., Zhou X. E., Hou L.*, et al.* An Intrinsic Agonist Mechanism for Activation of Glucagon-Like Peptide-1 Receptor by Its Extracellular Domain. Cell Discovery. 2016;2(1):16042.

509 Tomas A., Jones B., Leech C. New Insights into Beta-Cell Glp-1 Receptor and Camp Signaling. J Mol Biol. 2020;432(5):1347-66.
510 Wootten D., Christopoulos A., Sexton P. M. Emerging Paradigms in Gpcr Allostery: Implications for Drug Discovery. Nature Reviews Drug Discovery. 2013;12(8):630-44.
511 Koole C., Wootten D., Simms J.*, et al.* Allosteric Ligands of the Glucagon-Like Peptide 1 Receptor (Glp-1r) Differentially Modulate Endogenous and Exogenous Peptide Responses in a Pathway-Selective Manner: Implications for Drug Screening. Mol Pharmacol. 2010;78(3):456-65.
512 Jones B. J., Scopelliti R., Tomas A.*, et al.* Potent Prearranged Positive Allosteric Modulators of the Glucagon-Like Peptide-1 Receptor. ChemistryOpen. 2017;6(4):501-5.
513 Kawai K., Yokota C., Ohashi S.*, et al.* Evidence That Glucagon Stimulates Insulin Secretion through Its Own Receptor in Rats. Diabetologia. 1995;38(3):274-6.
514 Al-Zamel N., Al-Sabah S., Luqmani Y.*, et al.* A Dual Glp-1/Gip Receptor Agonist Does Not Antagonize Glucagon at Its Receptor but May Act as a Biased Agonist at the Glp-1 Receptor. Int J Mol Sci. 2019;20(14).
515 Schindelin J., Arganda-Carreras I., Frise E.*, et al.* Fiji: An Open-Source Platform for Biological-Image Analysis. Nature Methods. 2012;9(7):676-82.
516 Schneider C. A., Rasband W. S., Eliceiri K. W. Nih Image to Imagej: 25 Years of Image Analysis. Nature Methods. 2012;9(7):671-5.
517 Wan Q., Okashah N., Inoue A.*, et al.* Mini G Protein Probes for Active G Protein-Coupled Receptors (Gpcrs) in Live Cells. J Biol Chem. 2018;293(19):7466-73.
518 Shintani Y., Hayata-Takano A., Moriguchi K.*, et al.* B-Arrestin1 and 2 Differentially Regulate Pacap-Induced Pac1 Receptor Signaling and Trafficking. PLoS One. 2018;13(5):e0196946.
519 Kroeze W. K., Sassano M. F., Huang X. P.*, et al.* Presto-Tango as an Open-Source Resource for Interrogation of the Druggable Human Gpcrome. Nat Struct Mol Biol. 2015;22(5):362-9.
520 Zhong X., Chen Z., Chen Q.*, et al.* Novel Site-Specific Fatty Chain-Modified Glp-1 Receptor Agonist with Potent Antidiabetic Effects. Molecules. 2019;24(4).
521 Yin Y., Zhou X. E., Hou L.*, et al.* An Intrinsic Agonist Mechanism for Activation of Glucagon-Like Peptide-1 Receptor by Its Extracellular Domain. Cell Discov. 2016;2:16042.
522 Coopman K., Wallis R., Robb G.*, et al.* Residues within the Transmembrane Domain of the Glucagon-Like Peptide-1 Receptor Involved in Ligand Binding and Receptor Activation: Modelling the Ligand-Bound Receptor. Molecular Endocrinology. 2011;25(10):1804-18.
523 Hjorth S. A., Adelhorst K., Pedersen B. B.*, et al.* Glucagon and Glucagon-Like Peptide 1: Selective Receptor Recognition Via Distinct Peptide Epitopes. J Biol Chem. 1994;269(48):30121-4.
524 Adelhorst K., Hedegaard B. B., Knudsen L. B.*, et al.* Structure-Activity Studies of Glucagon-Like Peptide-1. J Biol Chem. 1994;269(9):6275-8.
525 Hager M. V., Clydesdale L., Gellman S. H.*, et al.* Characterization of Signal Bias at the Glp-1 Receptor Induced by Backbone Modification of Glp-1. Biochem Pharmacol. 2017;136:99-108.
526 Douillard C., Mention K., Dobbelaere D.*, et al.* Hypoglycaemia Related to Inherited Metabolic Diseases in Adults. Orphanet J Rare Dis. 2012;7.

527 Drucker D. J., Sherman S. I., Gorelick F. S.*, et al.* Incretin-Based Therapies for the Treatment of Type 2 Diabetes: Evaluation of the Risks and Benefits. Diabetes Care. 2010;33(2):428-33.
528 Kedia N. Treatment of Severe Diabetic Hypoglycemia with Glucagon: An Underutilized Therapeutic Approach. Diabetes Metab Syndr Obes. 2011;4:337-46.
529 Flakoll P. J., Borel M. J., Wentzel L. S.*, et al.* The Role of Glucagon in the Control of Protein and Amino Acid Metabolism in Vivo. Metabolism. 1994;43(12):1509-16.
530 Nair K. S., Halliday D., Matthews D. E.*, et al.* Hyperglucagonemia During Insulin Deficiency Accelerates Protein Catabolism. Am J Physiol. 1987;253(2 Pt 1):E208-13.
531 DeFronzo R. A., Jacot E., Jequier E.*, et al.* The Effect of Insulin on the Disposal of Intravenous Glucose: Results from Indirect Calorimetry and Hepatic and Femoral Venous Catheterization. Diabetes. 1981;30(12):1000-7.
532 Nilsson L. H., Hultman E. Liver and Muscle Glycogen in Man after Glucose and Fructose Infusion. Scand J Clin Lab Invest. 1974;33(1):5-10.
533 Pedersen C., Porsgaard T., Thomsen M.*, et al.* Sustained Effect of Glucagon on Body Weight and Blood Glucose: Assessed by Continuous Glucose Monitoring in Diabetic Rats. PLoS One. 2018;13(3):e0194468.
534 Davidson. I. W. F., Salter J. M., Best C. H. The Effect of Glucagon on the Metabolic Rate of Rats. The American Journal of Clinical Nutrition. 1960;8(5):540-6.
535 Weiser P. C., Grande F. Calorigenic Effects of Glucagon and Epinephrine in Anesthetized Dogs. Proc Soc Exp Biol Med. 1974;145(3):912-7.
536 Ingram D. L., Kaciuba-Uscilko H. Metabolic Effects of Glucagon in the Young Pig. Horm Metab Res. 1980;12(9):430-3.
537 Nair K. S. Hyperglucagonemia Increases Resting Metabolic Rate in Man During Insulin Deficiency. J Clin Endocrinol Metab. 1987;64(5):896-901.
538 Veldhorst M. A., Westerterp-Plantenga M. S., Westerterp K. R. Gluconeogenesis and Energy Expenditure after a High-Protein, Carbohydrate-Free Diet. Am J Clin Nutr. 2009;90(3):519-26.
539 Calles-Escandon J. Insulin Dissociates Hepatic Glucose Cycling and Glucagon-Induced Thermogenesis in Man. Metabolism. 1994;43(8):1000-5.
540 Nason S., Kim T., Antipenko J. P.*, et al.* Glucagon Regulates Energy Balance Via Fgf-21 Signaling in the Brain. Diabetes. 2018;67(Supplement 1):1806-P.
541 Van Schravendijk C. F., Foriers A., Hooghe-Peters E. L.*, et al.* Pancreatic Hormone Receptors on Islet Cells. Endocrinology. 1985;117(3):841-8.
542 Suga S., Kanno T., Nakano K.*, et al.* Glp-I(7-36) Amide Augments Ba2+ Current through L-Type Ca2+ Channel of Rat Pancreatic Beta-Cell in a Camp-Dependent Manner. Diabetes. 1997;46(11):1755-60.
543 Gromada J., Bokvist K., Ding W. G.*, et al.* Glucagon-Like Peptide 1 (7-36) Amide Stimulates Exocytosis in Human Pancreatic Beta-Cells by Both Proximal and Distal Regulatory Steps in Stimulus-Secretion Coupling. Diabetes. 1998;47(1):57-65.
544 Meloni A. R., DeYoung M. B., Lowe C.*, et al.* Glp-1 Receptor Activated Insulin Secretion from Pancreatic Beta-Cells: Mechanism and Glucose Dependence. Diabetes Obes Metab. 2013;15(1):15-27.
545 Janah L., Kjeldsen S., Galsgaard K. D.*, et al.* Glucagon Receptor Signaling and Glucagon Resistance. Int J Mol Sci. 2019;20(13).
546 Lv S., Qiu X., Li J.*, et al.* Glucagon-Induced Extracellular Camp Regulates Hepatic Lipid Metabolism. J Endocrinol. 2017;234(2):73-87.

547 Heibel S. K., Lopez G. Y., Panglao M.*, et al.* Transcriptional Regulation of N-Acetylglutamate Synthase. PLoS One. 2012;7(2):e29527.
548 Harrison C., Traynor J. R. The [35s]Gtpgammas Binding Assay: Approaches and Applications in Pharmacology. Life Sci. 2003;74(4):489-508.
549 Strange P. G. Use of the Gtpγs ([35s]Gtpγs and Eu-Gtpγs) Binding Assay for Analysis of Ligand Potency and Efficacy at G Protein-Coupled Receptors. Br J Pharmacol. 2010;161(6):1238-49.
550 Koval A., Kopein D., Purvanov V.*, et al.* Europium-Labeled Gtp as a General Nonradioactive Substitute for [(35)S]Gtpgammas in High-Throughput G Protein Studies. Anal Biochem. 2010;397(2):202-7.
551 Gales C., Rebois R. V., Hogue M.*, et al.* Real-Time Monitoring of Receptor and G-Protein Interactions in Living Cells. Nat Methods. 2005;2(3):177-84.
552 Masuho I., Martemyanov K. A., Lambert N. A. Monitoring G Protein Activation in Cells with Bret. Methods Mol Biol. 2015;1335:107-13.
553 Hall M. P., Unch J., Binkowski B. F.*, et al.* Engineered Luciferase Reporter from a Deep Sea Shrimp Utilizing a Novel Imidazopyrazinone Substrate. ACS Chem Biol. 2012;7(11):1848-57.
554 Boute N., Jockers R., Issad T. The Use of Resonance Energy Transfer in High-Throughput Screening: Bret Versus Fret. Trends Pharmacol Sci. 2002;23(8):351-4.
555 Pfleger K. D., Eidne K. A. Monitoring the Formation of Dynamic G-Protein-Coupled Receptor-Protein Complexes in Living Cells. Biochem J. 2005;385(Pt 3):625-37.
556 Lohse M. J., Nuber S., Hoffmann C. Fluorescence/Bioluminescence Resonance Energy Transfer Techniques to Study G-Protein-Coupled Receptor Activation and Signaling. Pharmacol Rev. 2012;64(2):299-336.
557 Dixon A. S., Schwinn M. K., Hall M. P.*, et al.* Nanoluc Complementation Reporter Optimized for Accurate Measurement of Protein Interactions in Cells. ACS Chem Biol. 2016;11(2):400-8.
558 Harper J. F., Brooker G. Femtomole Sensitive Radioimmunoassay for Cyclic Amp and Cyclic Gmp after 2'0 Acetylation by Acetic Anhydride in Aqueous Solution. J Cyclic Nucleotide Res. 1975;1(4):207-18.
559 Brooker G., Harper J. F., Terasaki W. L.*, et al.* Radioimmunoassay of Cyclic Amp and Cyclic Gmp. Adv Cyclic Nucleotide Res. 1979;10:1-33.
560 Mine T., Kojima I., Ogata E. Role of Calcium Fluxes in the Action of Glucagon on Glucose Metabolism in Rat Hepatocytes. Am J Physiol. 1993;265(1 Pt 1):G35-42.
561 Paredes R. M., Etzler J. C., Watts L. T.*, et al.* Chemical Calcium Indicators. Methods. 2008;46(3):143-51.
562 Matsu-ura T., Shirakawa H., Suzuki K. G. N.*, et al.* Dual-Fret Imaging of Ip3 and Ca2+ Revealed Ca2+-Induced Ip3 Production Maintains Long Lasting Ca2+ Oscillations in Fertilized Mouse Eggs. Scientific Reports. 2019;9(1):4829.
563 Pratt E. P., Salyer A. E., Guerra M. L.*, et al.* Ca2+ Influx through L-Type Ca2+ Channels and Ca2+-Induced Ca2+ Release Regulate Camp Accumulation and Epac1-Dependent Erk 1/2 Activation in Ins-1 Cells. Mol Cell Endocrinol. 2016;419:60-71.
564 Kang G., Holz G. G. Amplification of Exocytosis by Ca2+-Induced Ca2+ Release in Ins-1 Pancreatic Beta Cells. J Physiol. 2003;546(Pt 1):175-89.
565 Kim M. K., Cho J. H., Lee J. J.*, et al.* Proteomic Analysis of Ins-1 Rat Insulinoma Cells: Er Stress Effects and the Protective Role of Exenatide, a Glp-1 Receptor Agonist. PLoS One. 2015;10(3):e0120536.

566 Holz G. G., Leech C. A., Heller R. S.*, et al.* Camp-Dependent Mobilization of Intracellular Ca2+ Stores by Activation of Ryanodine Receptors in Pancreatic B-Cells: A Ca2+ Signaling System Stimulated by the Insulinotropic Hormone Glucagon-Like Peptide-1-(7–37). Journal of Biological Chemistry. 1999;274(20):14147-56.

567 Korge P., Weiss J. N. Thapsigargin Directly Induces the Mitochondrial Permeability Transition. Eur J Biochem. 1999;265(1):273-80.

568 van Unen J., Stumpf A. D., Schmid B.*, et al.* A New Generation of Fret Sensors for Robust Measurement of Gαi1, Gαi2 and Gαi3 Activation Kinetics in Single Cells. PLoS One. 2016;11(1):e0146789.

569 Kimple M. E., Neuman J. C., Linnemann A. K.*, et al.* Inhibitory G Proteins and Their Receptors: Emerging Therapeutic Targets for Obesity and Diabetes. Exp Mol Med. 2014;46:e102.

570 Rossi M., Zhu L., McMillin S. M.*, et al.* Hepatic Gi Signaling Regulates Whole-Body Glucose Homeostasis. J Clin Invest. 2018;128(2):746-59.

571 Burns D. L. Subunit Structure and Enzymic Activity of Pertussis Toxin. Microbiol Sci. 1988;5(9):285-7.

572 Luttrell L. M., Lefkowitz R. J. The Role of Beta-Arrestins in the Termination and Transduction of G-Protein-Coupled Receptor Signals. J Cell Sci. 2002;115(Pt 3):455-65.

573 Thomsen A. R. B., Plouffe B., Cahill T. J., 3rd*, et al.* Gpcr-G Protein-Beta-Arrestin Super-Complex Mediates Sustained G Protein Signaling. Cell. 2016;166(4):907-19.

574 Jean-Charles P. Y., Kaur S., Shenoy S. K. G Protein-Coupled Receptor Signaling through B-Arrestin-Dependent Mechanisms. J Cardiovasc Pharmacol. 2017;70(3):142-58.

575 Grundmann M., Merten N., Malfacini D.*, et al.* Lack of Beta-Arrestin Signaling in the Absence of Active G Proteins. Nat Commun. 2018;9(1):341.

576 Jung S. R., Seo J. B., Deng Y.*, et al.* Contributions of Protein Kinases and B-Arrestin to Termination of Protease-Activated Receptor 2 Signaling. J Gen Physiol. 2016;147(3):255-71.

577 Carr R., Schilling J., Song J.*, et al.* B-Arrestin–Biased Signaling through the B₂-Adrenergic Receptor Promotes Cardiomyocyte Contraction. Proceedings of the National Academy of Sciences. 2016;113(28):E4107-E16.

578 Jung S.-R., Kushmerick C., Seo J. B.*, et al.* Muscarinic Receptor Regulates Extracellular Signal Regulated Kinase by Two Modes of Arrestin Binding. Proceedings of the National Academy of Sciences. 2017;114(28):E5579-E88.

579 Hinz L., Ahles A., Ruprecht B.*, et al.* Two Serines in the Distal C-Terminus of the Human Ss1-Adrenoceptor Determine Ss-Arrestin2 Recruitment. PLoS One. 2017;12(5):e0176450.

580 Watts A. O., Verkaar F., van der Lee M. M.*, et al.* B-Arrestin Recruitment and G Protein Signaling by the Atypical Human Chemokine Decoy Receptor Ccx-Ckr. J Biol Chem. 2013;288(10):7169-81.

581 Donthamsetti P., Quejada J. R., Javitch J. A.*, et al.* Using Bioluminescence Resonance Energy Transfer (Bret) to Characterize Agonist-Induced Arrestin Recruitment to Modified and Unmodified G Protein-Coupled Receptors. Curr Protoc Pharmacol. 2015;70:2.14.1-2..

582 Fillion D., Devost D., Sleno R.*, et al.* Asymmetric Recruitment of B-Arrestin1/2 by the Angiotensin Ii Type I and Prostaglandin F2α Receptor Dimer. Front Endocrinol (Lausanne). 2019;10:162.

583 Pal K., Mathur M., Kumar P.*, et al.* Divergent Beta-Arrestin-Dependent Signaling Events Are Dependent Upon Sequences within G-Protein-Coupled Receptor C Termini. J Biol Chem. 2013;288(5):3265-74.

584 White C. W., Vanyai H. K., See H. B.*, et al.* Using Nanobret and Crispr/Cas9 to Monitor Proximity to a Genome-Edited Protein in Real-Time. Sci Rep. 2017;7(1):3187.

585 Machleidt T., Woodroofe C. C., Schwinn M. K.*, et al.* Nanobret--a Novel Bret Platform for the Analysis of Protein-Protein Interactions. ACS Chem Biol. 2015;10(8):1797-804.

586 Lu J., Li X., Wang Q.*, et al.* Dopamine D2 Receptor and B-Arrestin 2 Mediate Amyloid-B Elevation Induced by Anti-Parkinson's Disease Drugs, Levodopa and Piribedil, in Neuronal Cells. PLoS One. 2017;12(3):e0173240.

587 Reyes-Alcaraz A., Lee Y.-N., Yun S.*, et al.* Conformational Signatures in B-Arrestin2 Reveal Natural Biased Agonism at a G-Protein-Coupled Receptor. Communications Biology. 2018;1(1):128.

588 Dupuis N., Laschet C., Franssen D.*, et al.* Activation of the Orphan G Protein-Coupled Receptor Gpr27 by Surrogate Ligands Promotes Beta-Arrestin 2 Recruitment. Mol Pharmacol. 2017;91(6):595-608.

589 Barnea G., Strapps W., Herrada G.*, et al.* The Genetic Design of Signaling Cascades to Record Receptor Activation. Proc Natl Acad Sci U S A. 2008;105(1):64-9.

590 McGuinness D., Malikzay A., Visconti R.*, et al.* Characterizing Cannabinoid Cb2 Receptor Ligands Using Discoverx Pathhunter Beta-Arrestin Assay. J Biomol Screen. 2009;14(1):49-58.

591 Southern C., Cook J. M., Neetoo-Isseljee Z.*, et al.* Screening Beta-Arrestin Recruitment for the Identification of Natural Ligands for Orphan G-Protein-Coupled Receptors. J Biomol Screen. 2013;18(5):599-609.

592 Wang T., Li Z., Cvijic M. E.*, et al.* Measurement of Beta-Arrestin Recruitment for Gpcr Targets. In: Sittampalam GS, Grossman A, Brimacombe K, Arkin M, Auld D, Austin CP, et al., editors. Assay Guidance Manual. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2004.

593 Woo A. Y., Ge X. Y., Pan L.*, et al.* Discovery of Beta-Arrestin-Biased Beta2-Adrenoceptor Agonists from 2-Amino-2-Phenylethanol Derivatives. Acta Pharmacol Sin. 2019;40(8):1095-105.

594 Lee M. H., Appleton K. M., Strungs E. G.*, et al.* The Conformational Signature of B-Arrestin2 Predicts Its Trafficking and Signalling Functions. Nature. 2016;531(7596):665-8.

595 Nuber S., Zabel U., Lorenz K.*, et al.* B-Arrestin Biosensors Reveal a Rapid, Receptor-Dependent Activation/Deactivation Cycle. Nature. 2016;531(7596):661-4.

596 Smith J. S., Lefkowitz R. J., Rajagopal S. Biased Signalling: From Simple Switches to Allosteric Microprocessors. Nature Reviews Drug Discovery. 2018;17(4):243-60.

597 Griffin M. T., Figueroa K. W., Liller S.*, et al.* Estimation of Agonist Activity at G Protein-Coupled Receptors: Analysis of M₂ Muscarinic Receptor Signaling through G_{I/O},G_S, and G₁₅. Journal of Pharmacology and Experimental Therapeutics. 2007;321(3):1193-207.

598 Lynch A. M., Pathak N., Flatt Y. E.*, et al.* Comparison of Stability, Cellular, Glucose-Lowering and Appetite Supressing Effects of Oxyntomodulin Analogues Modified at the N-Terminus. Eur J Pharmacol. 2014;743:69-78.

599 Aroda V. R. A Review of Glp-1 Receptor Agonists: Evolution and Advancement, through the Lens of Randomised Controlled Trials. Diabetes Obes Metab. 2018;20 Suppl 1:22-33.

600 Bucheit J. D., Pamulapati L. G., Carter N.*, et al.* Oral Semaglutide: A Review of the First Oral Glucagon-Like Peptide 1 Receptor Agonist. Diabetes Technol Ther. 2020;22(1):10-8.

601 Heydenreich F. M., Vuckovic Z., Matkovic M.*, et al.* Stabilization of G Protein-Coupled Receptors by Point Mutations. Front Pharmacol. 2015;6:82.

602 Ostermaier M. K., Peterhans C., Jaussi R.*, et al.* Functional Map of Arrestin-1 at Single Amino Acid Resolution. Proc Natl Acad Sci U S A. 2014;111(5):1825-30.

603 Sato T., Kawasaki T., Mine S.*, et al.* Functional Role of the C-Terminal Amphipathic Helix 8 of Olfactory Receptors and Other G Protein-Coupled Receptors. Int J Mol Sci. 2016;17(11).

604 Mathi S. K., Chan Y., Li X.*, et al.* Scanning of the Glucagon-Like Peptide-1 Receptor Localizes G Protein-Activating Determinants Primarily to the N Terminus of the Third Intracellular Loop. Molecular Endocrinology. 1997;11(4):424-32.

605 Chabenne J., Chabenne M. D., Zhao Y.*, et al.* A Glucagon Analog Chemically Stabilized for Immediate Treatment of Life-Threatening Hypoglycemia. Mol Metab. 2014;3(3):293-300.

606 Yin Y., de Waal P. W., He Y.*, et al.* Rearrangement of a Polar Core Provides a Conserved Mechanism for Constitutive Activation of Class B G Protein-Coupled Receptors. J Biol Chem. 2017;292(24):9865-81.

607 Wingler L. M., Skiba M. A., McMahon C.*, et al.* Angiotensin and Biased Analogs Induce Structurally Distinct Active Conformations within a Gpcr. Science. 2020;367(6480):888-92.

608 Seyedabadi M., Ghahremani M. H., Albert P. R. Biased Signaling of G Protein Coupled Receptors (Gpcrs): Molecular Determinants of Gpcr/Transducer Selectivity and Therapeutic Potential. Pharmacol Ther. 2019;200:148-78.

609 Siu F. Y., He M., de Graaf C.*, et al.* Structure of the Human Glucagon Class B G-Protein-Coupled Receptor. Nature. 2013;499(7459):444-9.

610 Burgueno J., Pujol M., Monroy X.*, et al.* A Complementary Scale of Biased Agonism for Agonists with Differing Maximal Responses. Sci Rep. 2017;7(1):15389.

611 Stark K. L., Gross C., Richardson-Jones J.*, et al.* A Novel Conditional Knockout Strategy Applied to Serotonin Receptors. Handb Exp Pharmacol. 2007(178):347-63.

612 Wei P., Ahn Y. I., Housley P. R.*, et al.* Modulation of Hormone-Dependent Glucocorticoid Receptor Function Using a Tetracycline-Regulated Expression System. J Steroid Biochem Mol Biol. 1998;64(1-2):1-12.

613 Whalen E. J., Rajagopal S., Lefkowitz R. J. Therapeutic Potential of B-Arrestin- and G Protein-Biased Agonists. Trends Mol Med. 2011;17(3):126-39.

614 Reiter E., Lefkowitz R. J. Grks and Beta-Arrestins: Roles in Receptor Silencing, Trafficking and Signaling. Trends Endocrinol Metab. 2006;17(4):159-65.

615 Atwood B. K., Lopez J., Wager-Miller J.*, et al.* Expression of G Protein-Coupled Receptors and Related Proteins in Hek293, Att20, Bv2, and N18 Cell Lines as Revealed by Microarray Analysis. BMC Genomics. 2011;12:14.

616 Zhang R., Xie X. Tools for Gpcr Drug Discovery. Acta Pharmacol Sin. 2012;33(3):372-84.
617 Roed S. N., Wismann P., Underwood C. R.*, et al.* Real-Time Trafficking and Signaling of the Glucagon-Like Peptide-1 Receptor. Mol Cell Endocrinol. 2014;382(2):938-49.
618 Roed S. N., Nohr A. C., Wismann P.*, et al.* Functional Consequences of Glucagon-Like Peptide-1 Receptor Cross-Talk and Trafficking. J Biol Chem. 2015;290(2):1233-43.
619 Huvaere K., Skibsted L. H. Light-Induced Oxidation of Tryptophan and Histidine. Reactivity of Aromatic N-Heterocycles toward Triplet-Excited Flavins. J Am Chem Soc. 2009;131(23):8049-60.
620 Davies M. J., Truscott R. J. Photo-Oxidation of Proteins and Its Role in Cataractogenesis. J Photochem Photobiol B. 2001;63(1-3):114-25.
621 Ramnanan C. J., Edgerton D. S., Kraft G.*, et al.* Physiologic Action of Glucagon on Liver Glucose Metabolism. Diabetes Obes Metab. 2011;13 Suppl 1:118-25.
622 Song G., Pacini G., Ahrén B.*, et al.* Glucagon Increases Insulin Levels by Stimulating Insulin Secretion without Effect on Insulin Clearance in Mice. Peptides. 2017;88:74-9.
623 Vandamme T. F. Use of Rodents as Models of Human Diseases. J Pharm Bioallied Sci. 2014;6(1):2-9.
624 Bryant C. D. The Blessings and Curses of C57bl/6 Substrains in Mouse Genetic Studies. Ann N Y Acad Sci. 2011;1245:31-3.
625 Surwit R. S., Kuhn C. M., Cochrane C.*, et al.* Diet-Induced Type Ii Diabetes in C57bl/6j Mice. Diabetes. 1988;37(9):1163-7.
626 Fergusson G., Ethier M., Guevremont M.*, et al.* Defective Insulin Secretory Response to Intravenous Glucose in C57bl/6j Compared to C57bl/6n Mice. Mol Metab. 2014;3(9):848-54.
627 Freeman H. C., Hugill A., Dear N. T.*, et al.* Deletion of Nicotinamide Nucleotide Transhydrogenase: A New Quantitive Trait Locus Accounting for Glucose Intolerance in C57bl/6j Mice. Diabetes. 2006;55(7):2153-6.
628 Toye A. A., Lippiat J. D., Proks P.*, et al.* A Genetic and Physiological Study of Impaired Glucose Homeostasis Control in C57bl/6j Mice. Diabetologia. 2005;48(4):675-86.
629 Buganova M., Pelantova H., Holubova M.*, et al.* The Effects of Liraglutide in Mice with Diet-Induced Obesity Studied by Metabolomics. J Endocrinol. 2017;233(1):93-104.
630 Gabery S., Salinas C. G., Paulsen S. J.*, et al.* Semaglutide Lowers Body Weight in Rodents Via Distributed Neural Pathways. JCI Insight. 2020;5(6).
631 Elvert R., Herling A. W., Bossart M.*, et al.* Running on Mixed Fuel-Dual Agonistic Approach of Glp-1 and Gcg Receptors Leads to Beneficial Impact on Body Weight and Blood Glucose Control: A Comparative Study between Mice and Non-Human Primates. Diabetes Obes Metab. 2018;20(8):1836-51.
632 Svendsen B., Capozzi M. E., Nui J.*, et al.* Pharmacological Antagonism of the Incretin System Protects against Diet-Induced Obesity. Mol Metab. 2020;32:44-55.
633 Winzell M. S., Ahren B. The High-Fat Diet-Fed Mouse: A Model for Studying Mechanisms and Treatment of Impaired Glucose Tolerance and Type 2 Diabetes. Diabetes. 2004;53 Suppl 3:S215-9.
634 Heydemann A. An Overview of Murine High Fat Diet as a Model for Type 2 Diabetes Mellitus. J Diabetes Res. 2016;2016:2902351.
635 Speakman J. R. Use of High-Fat Diets to Study Rodent Obesity as a Model of Human Obesity. International Journal of Obesity. 2019;43(8):1491-2.

636 Hintze K. J., Benninghoff A. D., Cho C. E.*, et al.* Modeling the Western Diet for Preclinical Investigations. Adv Nutr. 2018;9(3):263-71.
637 Burcelin R., Crivelli V., Dacosta A.*, et al.* Heterogeneous Metabolic Adaptation of C57bl/6j Mice to High-Fat Diet. Am J Physiol Endocrinol Metab. 2002;282(4):E834-42.
638 Serino M., Luche E., Gres S.*, et al.* Metabolic Adaptation to a High-Fat Diet Is Associated with a Change in the Gut Microbiota. Gut. 2012;61(4):543-53.
639 Wang B., Chandrasekera P. C., Pippin J. J. Leptin- and Leptin Receptor-Deficient Rodent Models: Relevance for Human Type 2 Diabetes. Curr Diabetes Rev. 2014;10(2):131-45.
640 King A. J. The Use of Animal Models in Diabetes Research. Br J Pharmacol. 2012;166(3):877-94.
641 Leiter E. H. Selecting the "Right" Mouse Model for Metabolic Syndrome and Type 2 Diabetes Research. Methods Mol Biol. 2009;560:1-17.
642 Leiter E. H., Strobel M., O'Neill A.*, et al.* Comparison of Two New Mouse Models of Polygenic Type 2 Diabetes at the Jackson Laboratory, Noncnzo10lt/J and Tallyho/Jngj. J Diabetes Res. 2013;2013:165327.
643 Joost H. G., Schürmann A. The Genetic Basis of Obesity-Associated Type 2 Diabetes (Diabesity) in Polygenic Mouse Models. Mamm Genome. 2014;25(9-10):401-12.
644 Kim J. H., Stewart T. P., Soltani-Bejnood M.*, et al.* Phenotypic Characterization of Polygenic Type 2 Diabetes in Tallyho/Jngj Mice. J Endocrinol. 2006;191(2):437-46.
645 Burke S. J., Batdorf H. M., Burk D. H.*, et al.* Db/Db Mice Exhibit Features of Human Type 2 Diabetes That Are Not Present in Weight-Matched C57bl/6j Mice Fed a Western Diet. J Diabetes Res. 2017;2017:8503754.
646 Davis R. C., Castellani L. W., Hosseini M.*, et al.* Early Hepatic Insulin Resistance Precedes the Onset of Diabetes in Obese C57blks-<Em>Db/Db</Em> Mice. Diabetes. 2010;59(7):1616-25.
647 Srinivasan K., Ramarao P. Animal Models in Type 2 Diabetes Research: An Overview. Indian J Med Res. 2007;125(3):451-72.
648 Lindstrom P. The Physiology of Obese-Hyperglycemic Mice [Ob/Ob Mice]. ScientificWorldJournal. 2007;7:666-85.
649 Coleman D. L. Obese and Diabetes: Two Mutant Genes Causing Diabetes-Obesity Syndromes in Mice. Diabetologia. 1978;14(3):141-8.
650 Schoeller E. L., Chi M., Drury A.*, et al.* Leptin Monotherapy Rescues Spermatogenesis in Male Akita Type 1 Diabetic Mice. Endocrinology. 2014;155(8):2781-6.
651 Koch C. E., Lowe C., Pretz D.*, et al.* High-Fat Diet Induces Leptin Resistance in Leptin-Deficient Mice. J Neuroendocrinol. 2014;26(2):58-67.
652 Inagaki-Ohara K., Okamoto S., Takagi K.*, et al.* Leptin Receptor Signaling Is Required for High-Fat Diet-Induced Atrophic Gastritis in Mice. Nutrition & Metabolism. 2016;13(1):7.
653 Townsend K. L., Lorenzi M. M., Widmaier E. P. High-Fat Diet-Induced Changes in Body Mass and Hypothalamic Gene Expression in Wild-Type and Leptin-Deficient Mice. Endocrine. 2008;33(2):176-88.
654 Melez K. A., Harrison L. C., Gilliam J. N.*, et al.* Diabetes Is Associated with Autoimmunity in the New Zealand Obese (Nzo) Mouse. Diabetes. 1980;29(10):835-40.
655 Junger E., Herberg L., Jeruschke K.*, et al.* The Diabetes-Prone Nzo/Hl Strain. Ii. Pancreatic Immunopathology. Lab Invest. 2002;82(7):843-53.

656 Radaelli E., Santagostino S. F., Sellers R. S.*, et al.* Immune Relevant and Immune Deficient Mice: Options and Opportunities in Translational Research. Ilar j. 2018;59(3):211-46.
657 Petkova S. B., Yuan R., Tsaih S. W.*, et al.* Genetic Influence on Immune Phenotype Revealed Strain-Specific Variations in Peripheral Blood Lineages. Physiol Genomics. 2008;34(3):304-14.
658 Home Office. Annual Statistics of Scientific Procedures on Living Animals Great Britain 2018 2019 [Available from: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/835935/annual-statistics-scientific-procedures-living-animals-2018.pdf.
659 Iannaccone P. M., Jacob H. J. Rats! Dis Model Mech. 2009;2(5-6):206-10.
660 Marsh S. A., Dell'italia L. J., Chatham J. C. Interaction of Diet and Diabetes on Cardiovascular Function in Rats. Am J Physiol Heart Circ Physiol. 2009;296(2):H282-92.
661 Bugger H., Abel E. D. Rodent Models of Diabetic Cardiomyopathy. Dis Model Mech. 2009;2(9-10):454-66.
662 Pérez-Tilve D., González-Matías L., Aulinger B. A.*, et al.* Exendin-4 Increases Blood Glucose Levels Acutely in Rats by Activation of the Sympathetic Nervous System. Am J Physiol Endocrinol Metab. 2010;298(5):E1088-96.
663 Pick A., Clark J., Kubstrup C.*, et al.* Role of Apoptosis in Failure of Beta-Cell Mass Compensation for Insulin Resistance and Beta-Cell Defects in the Male Zucker Diabetic Fatty Rat. Diabetes. 1998;47(3):358-64.
664 Peterson R. G., Shaw W. N., Neel M.-A.*, et al.* Zucker Diabetic Fatty Rat as a Model for Non-Insulin-Dependent Diabetes Mellitus. ILAR Journal. 1990;32(3):16-9.
665 Yokoi N., Hoshino M., Hidaka S.*, et al.* A Novel Rat Model of Type 2 Diabetes: The Zucker Fatty Diabetes Mellitus Zfdm Rat. J Diabetes Res. 2013;2013:103731.
666 Zhao J., Zhang N., He M.*, et al.* Increased Beta-Cell Apoptosis and Impaired Insulin Signaling Pathway Contributes to the Onset of Diabetes in Oletf Rats. Cell Physiol Biochem. 2008;21(5-6):445-54.
667 Portha B., Giroix M. H., Serradas P.*, et al.* Beta-Cell Function and Viability in the Spontaneously Diabetic Gk Rat: Information from the Gk/Par Colony. Diabetes. 2001;50 Suppl 1:S89-93.
668 Miralles F., Portha B. Early Development of Beta-Cells Is Impaired in the Gk Rat Model of Type 2 Diabetes. Diabetes. 2001;50 Suppl 1:S84-8.
669 Östenson C. G., Khan A., Abdel-Halim S. M.*, et al.* Abnormal Insulin Secretion and Glucose Metabolism in Pancreatic Islets from the Spontaneously Diabetic Gk Rat. Diabetologia. 1993;36(1):3-8.
670 Lozano I., Van der Werf R., Bietiger W.*, et al.* High-Fructose and High-Fat Diet-Induced Disorders in Rats: Impact on Diabetes Risk, Hepatic and Vascular Complications. Nutrition & Metabolism. 2016;13(1):15.
671 Rossini A. A., Like A. A., Dulin W. E.*, et al.* Pancreatic Beta Cell Toxicity by Streptozotocin Anomers. Diabetes. 1977;26(12):1120-4.
672 Westermark P., Wernstedt C., O'Brien T. D.*, et al.* Islet Amyloid in Type 2 Human Diabetes Mellitus and Adult Diabetic Cats Contains a Novel Putative Polypeptide Hormone. Am J Pathol. 1987;127(3):414-7.

673 Wolf E., Braun-Reichhart C., Streckel E.*, et al.* Genetically Engineered Pig Models for Diabetes Research. Transgenic Res. 2014;23(1):27-38.
674 Lee M. S., Song K. D., Yang H. J.*, et al.* Development of a Type Ii Diabetic Mellitus Animal Model Using Micropig®. Lab Anim Res. 2012;28(3):205-8.
675 Larsen M. O., Rolin B. Use of the Gottingen Minipig as a Model of Diabetes, with Special Focus on Type 1 Diabetes Research. Ilar j. 2004;45(3):303-13.
676 Harwood H. J., Jr., Listrani P., Wagner J. D. Nonhuman Primates and Other Animal Models in Diabetes Research. J Diabetes Sci Technol. 2012;6(3):503-14.
677 Gotfredsen C. F., Molck A. M., Thorup I.*, et al.* The Human Glp-1 Analogs Liraglutide and Semaglutide: Absence of Histopathological Effects on the Pancreas in Nonhuman Primates. Diabetes. 2014;63(7):2486-97.
678 Moore M. C., Werner U., Smith M. S.*, et al.* Effect of the Glucagon-Like Peptide-1 Receptor Agonist Lixisenatide on Postprandial Hepatic Glucose Metabolism in the Conscious Dog. Am J Physiol Endocrinol Metab. 2013;305(12):E1473-82.
679 Werner U. Effects of the Glp-1 Receptor Agonist Lixisenatide on Postprandial Glucose and Gastric Emptying--Preclinical Evidence. J Diabetes Complications. 2014;28(1):110-4.
680 McGovern T. J. 208471origs000 - Tertiary Pharmacology/Toxicology Review. Food and Drug Administration; 2016.
681 Scrocchi L. A., Brown T. J., MaClusky N.*, et al.* Glucose Intolerance but Normal Satiety in Mice with a Null Mutation in the Glucagon-Like Peptide 1 Receptor Gene. Nat Med. 1996;2(11):1254-8.
682 Parker J. C., Andrews K. M., Allen M. R.*, et al.* Glycemic Control in Mice with Targeted Disruption of the Glucagon Receptor Gene. Biochem Biophys Res Commun. 2002;290(2):839-43.
683 Rivero-Gutierrez B., Haller A., Holland J.*, et al.* Deletion of the Glucagon Receptor Gene before and after Experimental Diabetes Reveals Differential Protection from Hyperglycemia. Mol Metab. 2018;17:28-38.
684 Pederson R. A., Satkunarajah M., McIntosh C. H.*, et al.* Enhanced Glucose-Dependent Insulinotropic Polypeptide Secretion and Insulinotropic Action in Glucagon-Like Peptide 1 Receptor -/- Mice. Diabetes. 1998;47(7):1046-52.
685 Longuet C., Robledo A. M., Dean E. D.*, et al.* Liver-Specific Disruption of the Murine Glucagon Receptor Produces A-Cell Hyperplasia: Evidence for a Circulating A-Cell Growth Factor. Diabetes. 2013;62(4):1196-205.
686 Kim H., Kim M., Im S. K.*, et al.* Mouse Cre-Loxp System: General Principles to Determine Tissue-Specific Roles of Target Genes. Lab Anim Res. 2018;34(4):147-59.
687 Metzger D., Chambon P. Site- and Time-Specific Gene Targeting in the Mouse. Methods. 2001;24(1):71-80.
688 Feil S., Valtcheva N., Feil R. Inducible Cre Mice. Methods Mol Biol. 2009;530:343-63.
689 Long M. A., Rossi F. M. Silencing Inhibits Cre-Mediated Recombination of the Z/Ap and Z/Eg Reporters in Adult Cells. PLoS One. 2009;4(5):e5435.
690 Liu J., Willet S. G., Bankaitis E. D.*, et al.* Non-Parallel Recombination Limits Cre-Loxp-Based Reporters as Precise Indicators of Conditional Genetic Manipulation. Genesis. 2013;51(6):436-42.
691 Borel F., Kay M. A., Mueller C. Recombinant Aav as a Platform for Translating the Therapeutic Potential of Rna Interference. Mol Ther. 2014;22(4):692-701.

692 McCarty D. M., Young S. M., Jr., Samulski R. J. Integration of Adeno-Associated Virus (Aav) and Recombinant Aav Vectors. Annu Rev Genet. 2004;38:819-45.
693 Daya S., Berns K. I. Gene Therapy Using Adeno-Associated Virus Vectors. Clin Microbiol Rev. 2008;21(4):583-93.
694 Hauck B., Xiao W. Characterization of Tissue Tropism Determinants of Adeno-Associated Virus Type 1. J Virol. 2003;77(4):2768-74.
695 Srivastava A. In Vivo Tissue-Tropism of Adeno-Associated Viral Vectors. Curr Opin Virol. 2016;21:75-80.
696 Wu Z., Yang H., Colosi P. Effect of Genome Size on Aav Vector Packaging. Mol Ther. 2010;18(1):80-6.
697 Chung J. Y., Ain Q. U., Song Y.*, et al.* Targeted Delivery of Crispr Interference System against Fabp4 to White Adipocytes Ameliorates Obesity, Inflammation, Hepatic Steatosis, and Insulin Resistance. Genome Res. 2019;29(9):1442-52.
698 Roh J. I., Lee J., Park S. U.*, et al.* Crispr-Cas9-Mediated Generation of Obese and Diabetic Mouse Models. Exp Anim. 2018;67(2):229-37.
699 Lodd E., Wiggenhauser L. M., Morgenstern J.*, et al.* The Combination of Loss of Glyoxalase1 and Obesity Results in Hyperglycemia. JCI Insight. 2019;4(12).
700 Zang L., Shimada Y., Nakayama H.*, et al.* Therapeutic Silencing of Centromere Protein X Ameliorates Hyperglycemia in Zebrafish and Mouse Models of Type 2 Diabetes Mellitus. Front Genet. 2019;10:693.
701 She J., Yuan Z., Wu Y.*, et al.* Targeting Erythropoietin Protects against Proteinuria in Type 2 Diabetic Patients and in Zebrafish. Mol Metab. 2018;8:189-202.
702 Ast J., Arvaniti A., Fine N. H. F.*, et al.* Super-Resolution Microscopy Compatible Fluorescent Probes Reveal Endogenous Glucagon-Like Peptide-1 Receptor Distribution and Dynamics. Nature Communications. 2020;11(1):467.
703 Cho S. W., Kim S., Kim Y.*, et al.* Analysis of Off-Target Effects of Crispr/Cas-Derived Rna-Guided Endonucleases and Nickases. Genome Res. 2014;24(1):132-41.
704 Mehravar M., Shirazi A., Nazari M.*, et al.* Mosaicism in Crispr/Cas9-Mediated Genome Editing. Dev Biol. 2019;445(2):156-62.
705 Stumvoll M., Mitrakou A., Pimenta W.*, et al.* Use of the Oral Glucose Tolerance Test to Assess Insulin Release and Insulin Sensitivity. Diabetes Care. 2000;23(3):295-301.
706 Chang T., Olson J. A., Proffitt R. T.*, et al.* Differences in Tissue Drug Concentrations Following Intravenous Versus Intraperitoneal Treatment with Amphotericin B Deoxycholate or Liposomal Amphotericin B. Med Mycol. 2010;48(2):430-5.
707 Watada S., Yu Y. M., Fischman A. J.*, et al.* Evaluation of Intragastric Vs Intraperitoneal Glucose Tolerance Tests in the Evaluation of Insulin Resistance in a Rodent Model of Burn Injury and Glucagon-Like Polypeptide-1 Treatment. J Burn Care Res. 2014;35(1):e66-72.
708 Reed D. R., Bachmanov A. A., Tordoff M. G. Forty Mouse Strain Survey of Body Composition. Physiol Behav. 2007;91(5):593-600.
709 Hoffler U., Hobbie K., Wilson R.*, et al.* Diet-Induced Obesity Is Associated with Hyperleptinemia, Hyperinsulinemia, Hepatic Steatosis, and Glomerulopathy in C57bl/6j Mice. Endocrine. 2009;36(2):311-25.
710 Jørgensen M. S., Tornqvist K. S., Hvid H. Calculation of Glucose Dose for Intraperitoneal Glucose Tolerance Tests in Lean and Obese Mice. J Am Assoc Lab Anim Sci. 2017;56(1):95-7.

711 Yen T. T., Stienmetz J., Simpson P. J. Blood Volume of Obese (Ob-Ob) and Diabetic (Db-Db) Mice. Proc Soc Exp Biol Med. 1970;133(1):307-8.
712 Okita K., Iwahashi H., Kozawa J.*, et al.* Usefulness of the Insulin Tolerance Test in Patients with Type 2 Diabetes Receiving Insulin Therapy. J Diabetes Investig. 2014;5(3):305-12.
713 Wang Q., Chen K., Liu R.*, et al.* Novel Glp-1 Fusion Chimera as Potent Long Acting Glp-1 Receptor Agonist. PLoS One. 2010;5(9):e12734.
714 Tang D., Tian H., Wu J.*, et al.* C-Terminal Site-Specific Pegylated Exendin-4 Analog: A Long-Acting Glucagon Like Peptide-1 Receptor Agonist, on Glycemic Control and Beta Cell Function in Diabetic Db/Db Mice. J Pharmacol Sci. 2018;138(1):23-30.
715 Ayala J. E., Bracy D. P., Malabanan C.*, et al.* Hyperinsulinemic-Euglycemic Clamps in Conscious, Unrestrained Mice. J Vis Exp. 2011(57).
716 DeFronzo R. A., Tobin J. D., Andres R. Glucose Clamp Technique: A Method for Quantifying Insulin Secretion and Resistance. Am J Physiol. 1979;237(3):E214-23.
717 Tam C. S., Xie W., Johnson W. D.*, et al.* Defining Insulin Resistance from Hyperinsulinemic-Euglycemic Clamps. Diabetes Care. 2012;35(7):1605-10.
718 Manley S. E., Stratton I. M., Clark P. M.*, et al.* Comparison of 11 Human Insulin Assays: Implications for Clinical Investigation and Research. Clin Chem. 2007;53(5):922-32.
719 Acharya A. P., Nafisi P. M., Gardner A.*, et al.* A Fluorescent Peroxidase Probe Increases the Sensitivity of Commercial Elisas by Two Orders of Magnitude. Chem Commun (Camb). 2013;49(88):10379-81.
720 Kagan A. Radioimmunoassay of Insulin. Semin Nucl Med. 1975;5(2):183-8.
721 Matthews D. R., Hosker J. P., Rudenski A. S.*, et al.* Homeostasis Model Assessment: Insulin Resistance and Beta-Cell Function from Fasting Plasma Glucose and Insulin Concentrations in Man. Diabetologia. 1985;28(7):412-9.
722 Wallace T. M., Levy J. C., Matthews D. R. Use and Abuse of Homa Modeling. Diabetes Care. 2004;27(6):1487-95.
723 Ellacott K. L., Morton G. J., Woods S. C.*, et al.* Assessment of Feeding Behavior in Laboratory Mice. Cell Metab. 2010;12(1):10-7.
724 Lerea J. S., Ring L. E., Hassouna R.*, et al.* Reducing Adiposity in a Critical Developmental Window Has Lasting Benefits in Mice. Endocrinology. 2016;157(2):666-78.
725 Nestoridi E., Kvas S., Kucharczyk J.*, et al.* Resting Energy Expenditure and Energetic Cost of Feeding Are Augmented after Roux-En-Y Gastric Bypass in Obese Mice. Endocrinology. 2012;153(5):2234-44.
726 Mashiko S., Ishihara A., Iwaasa H.*, et al.* A Pair-Feeding Study Reveals That a Y5 Antagonist Causes Weight Loss in Diet-Induced Obese Mice by Modulating Food Intake and Energy Expenditure. Mol Pharmacol. 2007;71(2):602-8.
727 Trayhurn P., Fuller L. The Development of Obesity in Genetically Diabetic-Obese (Db/Db) Mice Pair-Fed with Lean Siblings. The Importance of Thermoregulatory Thermogenesis. Diabetologia. 1980;19(2):148-53.
728 Kanoski S. E., Rupprecht L. E., Fortin S. M.*, et al.* The Role of Nausea in Food Intake and Body Weight Suppression by Peripheral Glp-1 Receptor Agonists, Exendin-4 and Liraglutide. Neuropharmacology. 2012;62(5-6):1916-27.

729 Kosinski J. R., Hubert J., Carrington P. E.*, et al.* The Glucagon Receptor Is Involved in Mediating the Body Weight-Lowering Effects of Oxyntomodulin. Obesity (Silver Spring). 2012;20(8):1566-71.

730 Deacon C. F., Johnsen A. H., Holst J. J. Degradation of Glucagon-Like Peptide-1 by Human Plasma in Vitro Yields an N-Terminally Truncated Peptide That Is a Major Endogenous Metabolite in Vivo. J Clin Endocrinol Metab. 1995;80(3):952-7.

731 Deacon C. F., Knudsen L. B., Madsen K.*, et al.* Dipeptidyl Peptidase Iv Resistant Analogues of Glucagon-Like Peptide-1 Which Have Extended Metabolic Stability and Improved Biological Activity. Diabetologia. 1998;41(3):271-8.

732 Schirra J., Sturm K., Leicht P.*, et al.* Exendin(9-39)Amide Is an Antagonist of Glucagon-Like Peptide-1(7-36)Amide in Humans. J Clin Invest. 1998;101(7):1421-30.

733 Unson C. G., Gurzenda E. M., Merrifield R. B. Biological Activities of Des-His1[Glu9]Glucagon Amide, a Glucagon Antagonist. Peptides. 1989;10(6):1171-7.

734 Cascieri M. A., Koch G. E., Ber E.*, et al.* Characterization of a Novel, Non-Peptidyl Antagonist of the Human Glucagon Receptor. J Biol Chem. 1999;274(13):8694-7.

735 Postic C., Shiota M., Niswender K. D.*, et al.* Dual Roles for Glucokinase in Glucose Homeostasis as Determined by Liver and Pancreatic Beta Cell-Specific Gene Knock-Outs Using Cre Recombinase. J Biol Chem. 1999;274(1):305-15.

736 Zhu L., Almaça J., Dadi P. K.*, et al.* B-Arrestin-2 Is an Essential Regulator of Pancreatic B-Cell Function under Physiological and Pathophysiological Conditions. Nat Commun. 2017;8:14295.

737 Irwin N., McClean P. L., Cassidy R. S.*, et al.* Comparison of the Anti-Diabetic Effects of Gip- and Glp-1-Receptor Activation in Obese Diabetic (Ob/Ob) Mice: Studies with Dpp Iv Resistant N-Acgip and Exendin(1-39)Amide. Diabetes Metab Res Rev. 2007;23(7):572-9.

738 Gault V. A., O'Harte F. P., Harriott P.*, et al.* Degradation, Cyclic Adenosine Monophosphate Production, Insulin Secretion, and Glycemic Effects of Two Novel N-Terminal Ala2-Substituted Analogs of Glucose-Dependent Insulinotropic Polypeptide with Preserved Biological Activity in Vivo. Metabolism. 2003;52(6):679-87.

739 Gu W., Winters K. A., Motani A. S.*, et al.* Glucagon Receptor Antagonist-Mediated Improvements in Glycemic Control Are Dependent on Functional Pancreatic Glp-1 Receptor. Am J Physiol Endocrinol Metab. 2010;299(4):E624-32.

740 Yan H., Gu W., Yang J.*, et al.* Fully Human Monoclonal Antibodies Antagonizing the Glucagon Receptor Improve Glucose Homeostasis in Mice and Monkeys. J Pharmacol Exp Ther. 2009;329(1):102-11.

741 Nance K. D., Days E. L., Weaver C. D.*, et al.* Discovery of a Novel Series of Orally Bioavailable and Cns Penetrant Glucagon-Like Peptide-1 Receptor (Glp-1r) Noncompetitive Antagonists Based on a 1,3-Disubstituted-7-Aryl-5,5-Bis(Trifluoromethyl)-5,8-Dihydropyrimido[4,5-D]Pyrim Idine-2,4(1h,3h)-Dione Core. J Med Chem. 2017;60(4):1611-6.

742 Pettersson U. S., Waldén T. B., Carlsson P. O.*, et al.* Female Mice Are Protected against High-Fat Diet Induced Metabolic Syndrome and Increase the Regulatory T Cell Population in Adipose Tissue. PLoS One. 2012;7(9):e46057.

743 Reynolds T. H., Dalton A., Calzini L.*, et al.* The Impact of Age and Sex on Body Composition and Glucose Sensitivity in C57bl/6j Mice. Physiol Rep. 2019;7(3):e13995.

744 Stubbins R. E., Holcomb V. B., Hong J.*, et al.* Estrogen Modulates Abdominal Adiposity and Protects Female Mice from Obesity and Impaired Glucose Tolerance. Eur J Nutr. 2012;51(7):861-70.
745 Basu R., Dalla Man C., Campioni M.*, et al.* Effects of Age and Sex on Postprandial Glucose Metabolism: Differences in Glucose Turnover, Insulin Secretion, Insulin Action, and Hepatic Insulin Extraction. Diabetes. 2006;55(7):2001-14.
746 Amiel S. A., Maran A., Powrie J. K.*, et al.* Gender Differences in Counterregulation to Hypoglycaemia. Diabetologia. 1993;36(5):460-4.
747 Anichini R., Cosimi S., Di Carlo A.*, et al.* Gender Difference in Response Predictors after 1-Year Exenatide Therapy Twice Daily in Type 2 Diabetic Patients: A Real World Experience. Diabetes Metab Syndr Obes. 2013;6:123-9.
748 Zhang Y., Parajuli K. R., Fava G. E.*, et al.* Glp-1 Receptor in Pancreatic A-Cells Regulates Glucagon Secretion in a Glucose-Dependent Bidirectional Manner. Diabetes. 2019;68(1):34-44.
749 Magnuson M. A., Osipovich A. B. Pancreas-Specific Cre Driver Lines and Considerations for Their Prudent Use. Cell Metab. 2013;18(1):9-20.
750 Honig G., Liou A., Berger M.*, et al.* Precise Pattern of Recombination in Serotonergic and Hypothalamic Neurons in a Pdx1-Cre Transgenic Mouse Line. J Biomed Sci. 2010;17:82.
751 Schonhoff S. E., Giel-Moloney M., Leiter A. B. Neurogenin 3-Expressing Progenitor Cells in the Gastrointestinal Tract Differentiate into Both Endocrine and Non-Endocrine Cell Types. Dev Biol. 2004;270(2):443-54.
752 Saper C. B., Chou T. C., Elmquist J. K. The Need to Feed: Homeostatic and Hedonic Control of Eating. Neuron. 2002;36(2):199-211.
753 Benton D., Young H. A. Reducing Calorie Intake May Not Help You Lose Body Weight. Perspect Psychol Sci. 2017;12(5):703-14.
754 Roedel A., Storch C., Holsboer F.*, et al.* Effects of Light or Dark Phase Testing on Behavioural and Cognitive Performance in Dba Mice. Lab Anim. 2006;40(4):371-81.
755 Schilperoort M., van den Berg R., Dollé M. E. T.*, et al.* Time-Restricted Feeding Improves Adaptation to Chronically Alternating Light-Dark Cycles. Scientific Reports. 2019;9(1):7874.
756 Huber J. D., Egleton R. D., Davis T. P. Molecular Physiology and Pathophysiology of Tight Junctions in the Blood-Brain Barrier. Trends Neurosci. 2001;24(12):719-25.
757 Bray G. M. Exenatide. Am J Health Syst Pharm. 2006;63(5):411-8.
758 Göke R., Fehmann H. C., Linn T.*, et al.* Exendin-4 Is a High Potency Agonist and Truncated Exendin-(9-39)-Amide an Antagonist at the Glucagon-Like Peptide 1-(7-36)-Amide Receptor of Insulin-Secreting Beta-Cells. J Biol Chem. 1993;268(26):19650-5.
759 Egan J. M., Clocquet A. R., Elahi D. The Insulinotropic Effect of Acute Exendin-4 Administered to Humans: Comparison of Nondiabetic State to Type 2 Diabetes. The Journal of Clinical Endocrinology & Metabolism. 2002;87(3):1282-90.
760 Kendall D. M., Riddle M. C., Rosenstock J.*, et al.* Effects of Exenatide (Exendin-4) on Glycemic Control over 30 Weeks in Patients with Type 2 Diabetes Treated with Metformin and a Sulfonylurea. Diabetes Care. 2005;28(5):1083-91.
761 Thorkildsen C., Neve S., Larsen B. D.*, et al.* Glucagon-Like Peptide 1 Receptor Agonist Zp10a Increases Insulin Mrna Expression and Prevents Diabetic Progression in Db/Db Mice. J Pharmacol Exp Ther. 2003;307(2):490-6.

762 Baker D. E., Levien T. L. Lixisenatide. Hosp Pharm. 2017;52(1):65-80.
763 Christensen M., Knop F. K., Holst J. J.*, et al.* Lixisenatide, a Novel Glp-1 Receptor Agonist for the Treatment of Type 2 Diabetes Mellitus. IDrugs. 2009;12(8):503-13.
764 Barnett A. H. Lixisenatide: Evidence for Its Potential Use in the Treatment of Type 2 Diabetes. Core Evid. 2011;6:67-79.
765 Becker R. H., Stechl J., Msihid J.*, et al.* Lixisenatide Resensitizes the Insulin-Secretory Response to Intravenous Glucose Challenge in People with Type 2 Diabetes--a Study in Both People with Type 2 Diabetes and Healthy Subjects. Diabetes Obes Metab. 2014;16(9):793-800.
766 Meneilly G. S., Roy-Duval C., Alawi H.*, et al.* Lixisenatide Therapy in Older Patients with Type 2 Diabetes Inadequately Controlled on Their Current Antidiabetic Treatment: The Getgoal-O Randomized Trial. Diabetes Care. 2017;40(4):485-93.
767 Ratner R. E., Rosenstock J., Boka G. Dose-Dependent Effects of the Once-Daily Glp-1 Receptor Agonist Lixisenatide in Patients with Type 2 Diabetes Inadequately Controlled with Metformin: A Randomized, Double-Blind, Placebo-Controlled Trial. Diabet Med. 2010;27(9):1024-32.
768 Rosenstock J., Raccah D., Korányi L.*, et al.* Efficacy and Safety of Lixisenatide Once Daily Versus Exenatide Twice Daily in Type 2 Diabetes Inadequately Controlled on Metformin: A 24-Week, Randomized, Open-Label, Active-Controlled Study (Getgoal-X). Diabetes Care. 2013;36(10):2945-51.
769 Bech E. M., Pedersen S. L., Jensen K. J. Chemical Strategies for Half-Life Extension of Biopharmaceuticals: Lipidation and Its Alternatives. ACS Med Chem Lett. 2018;9(7):577-80.
770 Schultz H. S., Østergaard S., Sidney J.*, et al.* The Effect of Acylation with Fatty Acids and Other Modifications on Hla Class Ii:Peptide Binding and T Cell Stimulation for Three Model Peptides. PLoS One. 2018;13(5):e0197407.
771 Yu M., Benjamin M. M., Srinivasan S.*, et al.* Battle of Glp-1 Delivery Technologies. Adv Drug Deliv Rev. 2018;130:113-30.
772 Knudsen L. B., Nielsen P. F., Huusfeldt P. O.*, et al.* Potent Derivatives of Glucagon-Like Peptide-1 with Pharmacokinetic Properties Suitable for Once Daily Administration. J Med Chem. 2000;43(9):1664-9.
773 Jackson S. H., Martin T. S., Jones J. D.*, et al.* Liraglutide (Victoza): The First Once-Daily Incretin Mimetic Injection for Type-2 Diabetes. P t. 2010;35(9):498-529.
774 Madsen K., Knudsen L. B., Agersoe H.*, et al.* Structure-Activity and Protraction Relationship of Long-Acting Glucagon-Like Peptide-1 Derivatives: Importance of Fatty Acid Length, Polarity, and Bulkiness. J Med Chem. 2007;50(24):6126-32.
775 Jensen L., Helleberg H., Roffel A.*, et al.* Absorption, Metabolism and Excretion of the Glp-1 Analogue Semaglutide in Humans and Nonclinical Species. Eur J Pharm Sci. 2017;104:31-41.
776 Bush M. A., Matthews J. E., De Boever E. H.*, et al.* Safety, Tolerability, Pharmacodynamics and Pharmacokinetics of Albiglutide, a Long-Acting Glucagon-Like Peptide-1 Mimetic, in Healthy Subjects. Diabetes Obes Metab. 2009;11(5):498-505.
777 Jimenez-Solem E., Rasmussen M. H., Christensen M.*, et al.* Dulaglutide, a Long-Acting Glp-1 Analog Fused with an Fc Antibody Fragment for the Potential Treatment of Type 2 Diabetes. Curr Opin Mol Ther. 2010;12(6):790-7.

778 Matthews J. E., Stewart M. W., De Boever E. H.*, et al.* Pharmacodynamics, Pharmacokinetics, Safety, and Tolerability of Albiglutide, a Long-Acting Glucagon-Like Peptide-1 Mimetic, in Patients with Type 2 Diabetes. The Journal of Clinical Endocrinology & Metabolism. 2008;93(12):4810-7.

779 Geiser J. S., Heathman M. A., Cui X.*, et al.* Clinical Pharmacokinetics of Dulaglutide in Patients with Type 2 Diabetes: Analyses of Data from Clinical Trials. Clinical Pharmacokinetics. 2016;55(5):625-34.

780 Nauck M. A., Ratner R. E., Kapitza C.*, et al.* Treatment with the Human Once-Weekly Glucagon-Like Peptide-1 Analog Taspoglutide in Combination with Metformin Improves Glycemic Control and Lowers Body Weight in Patients with Type 2 Diabetes Inadequately Controlled with Metformin Alone: A Double-Blind Placebo-Controlled Study. Diabetes Care. 2009;32(7):1237-43.

781 Mentlein R., Gallwitz B., Schmidt W. E. Dipeptidyl-Peptidase Iv Hydrolyses Gastric Inhibitory Polypeptide, Glucagon-Like Peptide-1(7-36)Amide, Peptide Histidine Methionine and Is Responsible for Their Degradation in Human Serum. Eur J Biochem. 1993;214(3):829-35.

782 Ratner R., Nauck M., Kapitza C.*, et al.* Safety and Tolerability of High Doses of Taspoglutide, a Once-Weekly Human Glp-1 Analogue, in Diabetic Patients Treated with Metformin: A Randomized Double-Blind Placebo-Controlled Study. Diabet Med. 2010;27(5):556-62.

783 Rosenstock J., Balas B., Charbonnel B.*, et al.* The Fate of Taspoglutide, a Weekly Glp-1 Receptor Agonist, Versus Twice-Daily Exenatide for Type 2 Diabetes: The T-Emerge 2 Trial. Diabetes Care. 2013;36(3):498-504.

784 Solloway M. J., Madjidi A., Gu C.*, et al.* Glucagon Couples Hepatic Amino Acid Catabolism to Mtor-Dependent Regulation of A-Cell Mass. Cell Rep. 2015;12(3):495-510.

785 Thiessen S. E., Derde S., Derese I.*, et al.* Role of Glucagon in Catabolism and Muscle Wasting of Critical Illness and Modulation by Nutrition. Am J Respir Crit Care Med. 2017;196(9):1131-43.

786 Brillon D. J., Zheng B., Campbell R. G.*, et al.* Effect of Cortisol on Energy Expenditure and Amino Acid Metabolism in Humans. Am J Physiol. 1995;268(3 Pt 1):E501-13.

787 Duren D. L., Sherwood R. J., Czerwinski S. A.*, et al.* Body Composition Methods: Comparisons and Interpretation. J Diabetes Sci Technol. 2008;2(6):1139-46.

788 Nuttall F. Q. Body Mass Index: Obesity, Bmi, and Health: A Critical Review. Nutr Today. 2015;50(3):117-28.

789 Pietrobelli A., Formica C., Wang Z.*, et al.* Dual-Energy X-Ray Absorptiometry Body Composition Model: Review of Physical Concepts. Am J Physiol. 1996;271(6 Pt 1):E941-51.

790 Mystkowski P., Shankland E., Schreyer S. A.*, et al.* Validation of Whole-Body Magnetic Resonance Spectroscopy as a Tool to Assess Murine Body Composition. Int J Obes Relat Metab Disord. 2000;24(6):719-24.

791 Halldorsdottir S., Carmody J., Boozer C. N.*, et al.* Reproducibility and Accuracy of Body Composition Assessments in Mice by Dual Energy X-Ray Absorptiometry and Time Domain Nuclear Magnetic Resonance. Int J Body Compos Res. 2009;7(4):147-54.

792 Nagy T. R., Clair A. L. Precision and Accuracy of Dual-Energy X-Ray Absorptiometry for Determining in Vivo Body Composition of Mice. Obes Res. 2000;8(5):392-8.

793 Tinsley F. C., Taicher G. Z., Heiman M. L. Evaluation of a Quantitative Magnetic Resonance Method for Mouse Whole Body Composition Analysis. Obes Res. 2004;12(1):150-60.
794 Heppner K. M., Marks S., Holland J.*, et al.* Contribution of Brown Adipose Tissue Activity to the Control of Energy Balance by Glp-1 Receptor Signalling in Mice. Diabetologia. 2015;58(9):2124-32.
795 Polyzos S. A., Kountouras J., Mantzoros C. S. Obesity and Nonalcoholic Fatty Liver Disease: From Pathophysiology to Therapeutics. Metabolism. 2019;92:82-97.
796 Sarwar R., Pierce N., Koppe S. Obesity and Nonalcoholic Fatty Liver Disease: Current Perspectives. Diabetes Metab Syndr Obes. 2018;11:533-42.
797 Tushuizen M. E., Bunck M. C., Pouwels P. J.*, et al.* Incretin Mimetics as a Novel Therapeutic Option for Hepatic Steatosis. Liver Int. 2006;26(8):1015-7.
798 Dutour A., Abdesselam I., Ancel P.*, et al.* Exenatide Decreases Liver Fat Content and Epicardial Adipose Tissue in Patients with Obesity and Type 2 Diabetes: A Prospective Randomized Clinical Trial Using Magnetic Resonance Imaging and Spectroscopy. Diabetes Obes Metab. 2016;18(9):882-91.
799 Fan H., Pan Q., Xu Y.*, et al.* Exenatide Improves Type 2 Diabetes Concomitant with Non-Alcoholic Fatty Liver Disease. Arq Bras Endocrinol Metabol. 2013;57(9):702-8.
800 Khoo J., Hsiang J., Taneja R.*, et al.* Comparative Effects of Liraglutide 3 Mg Vs Structured Lifestyle Modification on Body Weight, Liver Fat and Liver Function in Obese Patients with Non-Alcoholic Fatty Liver Disease: A Pilot Randomized Trial. Diabetes Obes Metab. 2017;19(12):1814-7.
801 More V. R., Lao J., McLaren D. G.*, et al.* Glucagon Like Receptor 1/ Glucagon Dual Agonist Acutely Enhanced Hepatic Lipid Clearance and Suppressed De Novo Lipogenesis in Mice. PLoS One. 2017;12(10):e0186586.
802 Beaton M., Guionaud S., Conway J. P.*, et al.* Medi0382, a Glp-1/Glucagon Receptor Dual Agonist, Dramatically Reduces Hepatic Collagen in a Mouse Model of Nash. Diabetes. 2018;67(Supplement 1):1841-P.
803 Armstrong M. J., Hull D., Guo K.*, et al.* Glucagon-Like Peptide 1 Decreases Lipotoxicity in Non-Alcoholic Steatohepatitis. J Hepatol. 2016;64(2):399-408.
804 Mells J. E., Fu P. P., Sharma S.*, et al.* Glp-1 Analog, Liraglutide, Ameliorates Hepatic Steatosis and Cardiac Hypertrophy in C57bl/6j Mice Fed a Western Diet. Am J Physiol Gastrointest Liver Physiol. 2012;302(2):G225-35.
805 Maida A., Lovshin J. A., Baggio L. L.*, et al.* The Glucagon-Like Peptide-1 Receptor Agonist Oxyntomodulin Enhances Beta-Cell Function but Does Not Inhibit Gastric Emptying in Mice. Endocrinology. 2008;149(11):5670-8.
806 Horowitz M., Aroda V. R., Han J.*, et al.* Upper and/or Lower Gastrointestinal Adverse Events with Glucagon-Like Peptide-1 Receptor Agonists: Incidence and Consequences. Diabetes Obes Metab. 2017;19(5):672-81.
807 Charron M. J., Vuguin P. M. Lack of Glucagon Receptor Signaling and Its Implications Beyond Glucose Homeostasis. J Endocrinol. 2015;224(3):R123-30.
808 Ayala J. E., Bracy D. P., James F. D.*, et al.* Glucagon-Like Peptide-1 Receptor Knockout Mice Are Protected from High-Fat Diet-Induced Insulin Resistance. Endocrinology. 2010;151(10):4678-87.
809 Chen M., Mema E., Kelleher J.*, et al.* Absence of the Glucagon-Like Peptide-1 Receptor Does Not Affect the Metabolic Phenotype of Mice with Liver-Specific G(S)A Deficiency. Endocrinology. 2011;152(9):3343-50.

810 Jessen L., Aulinger B. A., Hassel J. L*., et al.* Suppression of Food Intake by Glucagon-Like Peptide-1 Receptor Agonists: Relative Potencies and Role of Dipeptidyl Peptidase-4. Endocrinology. 2012;153(12):5735-45.

811 Radziszewska E., Bojanowska E. Effects of Glucagon-Like Peptide-1 Receptor Stimulation and Blockade on Food Consumption and Body Weight in Rats Treated with a Cannabinoid Cb1 Receptor Agonist Win 55,212-2. Med Sci Monit Basic Res. 2013;19:6-11.

812 Rodriquez de Fonseca F., Navarro M., Alvarez E*., et al.* Peripheral Versus Central Effects of Glucagon-Like Peptide-1 Receptor Agonists on Satiety and Body Weight Loss in Zucker Obese Rats. Metabolism. 2000;49(6):709-17.

813 Asnake S., Modig C., Olsson P. E. Species Differences in Ligand Interaction and Activation of Estrogen Receptors in Fish and Human. J Steroid Biochem Mol Biol. 2019;195:105450.

814 Ray T. D., Mekasha S., Liang Y*., et al.* Species-Specific Differences in Regulation of Macrophage Inflammation by the C3a-C3a Receptor Axis. Innate Immun. 2018;24(1):66-78.

815 Li Z., Liang Y., Xia N*., et al.* Liraglutide Reduces Body Weight by Upregulation of Adenylate Cyclase 3. Nutrition & Diabetes. 2017;7(5):e265-e.

816 Zhou K., Wolski K., Malin S. K*., et al.* Impact of Weight Loss Trajectory Following Randomization to Bariatric Surgery on Long-Term Diabetes Glycemic and Cardiometabolic Parameters. Endocr Pract. 2019;25(6):572-9.

817 Swarbrick M. M., Austrheim-Smith I. T., Stanhope K. L*., et al.* Circulating Concentrations of High-Molecular-Weight Adiponectin Are Increased Following Roux-En-Y Gastric Bypass Surgery. Diabetologia. 2006;49(11):2552-8.

818 Wan Y., Bao X., Huang J*., et al.* Novel Glp-1 Analog Supaglutide Reduces Hfd-Induced Obesity Associated with Increased Ucp-1 in White Adipose Tissue in Mice. Front Physiol. 2017;8:294.

819 Hong Y., Lee J. H., Jeong K. W*., et al.* Amelioration of Muscle Wasting by Glucagon-Like Peptide-1 Receptor Agonist in Muscle Atrophy. J Cachexia Sarcopenia Muscle. 2019;10(4):903-18.

820 Courcoulas A. P., Christian N. J., Belle S. H*., et al.* Weight Change and Health Outcomes at 3 Years after Bariatric Surgery among Individuals with Severe Obesity. Jama. 2013;310(22):2416-25.

821 Courcoulas A. P., King W. C., Belle S. H*., et al.* Seven-Year Weight Trajectories and Health Outcomes in the Longitudinal Assessment of Bariatric Surgery (Labs) Study. JAMA Surg. 2018;153(5):427-34.

822 Bruschi Kelles S. M., Diniz M. F., Machado C. J*., et al.* Mortality Rate after Open Roux-in-Y Gastric Bypass: A 10-Year Follow-Up. Braz J Med Biol Res. 2014;47(7):617-25.

823 Moon R. C., Kreimer F., Teixeira A. F*., et al.* Morbidity Rates and Weight Loss after Roux-En-Y Gastric Bypass, Sleeve Gastrectomy, and Adjustable Gastric Banding in Patients Older Than 60 Years Old: Which Procedure to Choose? Obes Surg. 2016;26(4):730-6.

824 Finan B., Clemmensen C., Müller T. D. Emerging Opportunities for the Treatment of Metabolic Diseases: Glucagon-Like Peptide-1 Based Multi-Agonists. Mol Cell Endocrinol. 2015;418 Pt 1:42-54.

825 Elvert R., Bossart M., Zhang B.*, et al.* Weight Loss Outcomes with Species-Specific Dual Glp-1r/Gcgr Agonists in Animal Models. Diabetes. 2018;67(Supplement 1):2028-P.

826 Pedersen M. F., Wróbel T. M., Märcher-Rørsted E.*, et al.* Biased Agonism of Clinically Approved M-Opioid Receptor Agonists and Trv130 Is Not Controlled by Binding and Signaling Kinetics. Neuropharmacology. 2020;166:107718.

827 Buenaventura T., Bitsi S., Laughlin W. E.*, et al.* Agonist-Induced Membrane Nanodomain Clustering Drives Glp-1 Receptor Responses in Pancreatic Beta Cells. PLOS Biology. 2019;17(8):e3000097.

828 Pickford P. J., Lucey M. A., Fang Z.*, et al.* Signalling, Trafficking and Glucoregulatory Properties of Glucagon-Like Peptide-1 Receptor Agonists Exendin-4 and Lixisenatide. British Pharmacological Society. 2020.

829 Conarello S. L., Jiang G., Mu J.*, et al.* Glucagon Receptor Knockout Mice Are Resistant to Diet-Induced Obesity and Streptozotocin-Mediated Beta Cell Loss and Hyperglycaemia. Diabetologia. 2007;50(1):142-50.

830 Lee Y., Wang M. Y., Du X. Q.*, et al.* Glucagon Receptor Knockout Prevents Insulin-Deficient Type 1 Diabetes in Mice. Diabetes. 2011;60(2):391-7.

831 Pierantonelli I., Svegliati-Baroni G. Nonalcoholic Fatty Liver Disease: Basic Pathogenetic Mechanisms in the Progression from Nafld to Nash. Transplantation. 2019;103(1):e1-e13.

832 Jia X., Alam M., Ye Y.*, et al.* Glp-1 Receptor Agonists and Cardiovascular Disease: A Meta-Analysis of Recent Cardiac Outcome Trials. Cardiovasc Drugs Ther. 2018;32(1):65-72.

833 Salcedo I., Tweedie D., Li Y.*, et al.* Neuroprotective and Neurotrophic Actions of Glucagon-Like Peptide-1: An Emerging Opportunity to Treat Neurodegenerative and Cerebrovascular Disorders. Br J Pharmacol. 2012;166(5):1586-99.

834 Hay M., Thomas D. W., Craighead J. L.*, et al.* Clinical Development Success Rates for Investigational Drugs. Nature Biotechnology. 2014;32(1):40-51.