

Physiological effects of biased glucagon-like peptide-1 receptor agonists

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

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2019

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Abstract

Glucagon-like peptide-1 receptor agonists (GLP-1RAs) are an effective drug class used in the treatment of type 2 diabetes mellitus (T2DM) and have recently been licensed for obesity independently of diabetes. Their beneficial effects include the potentiation of glucose-stimulated insulin secretion, enhancement of pancreatic β -cell function, and promotion of weight loss. However, they are associated with gastrointestinal side effects, of which nausea is the most common. Published data from the early stage clinical development of approved GLP-1RAs repeatedly demonstrate that doses in excess of those subsequently licensed have greater glycaemic efficacy but unacceptable tolerability. This suggests that the maximum therapeutic potential of GLP-1R agonism has not yet been reached, offering scope for its optimisation.

Recent work suggests that biased agonism – the ability of receptor ligands to selectively activate particular intracellular pathways – can be leveraged to therapeutically optimise GLP-1RAs. Exendin-4-based GLP-1RAs biased towards G-protein signalling over β -arrestin recruitment exhibited enhanced anti-hyperglycaemic effects with reduced signs of nausea. Intriguingly, the superior glycaemic efficacy of G-protein-biased agonists did not translate into commensurate increases in anorectic efficacy, suggesting a divergence between the effects of biased GLP-1RAs upon glucoregulation *versus* appetite suppression.

This project first confirmed the existence of a physiological divergence between the glycaemic and anorectic effects of exendin-4, G-protein-biased exendin-phe1, and β -arrestin-biased exendin-asp3, and demonstrated the impact of this on the “therapeutic window” of these ligands. Two testable hypotheses were then offered for the observed physiological divergence: differences in the *access* of the biased GLP-1RAs to CNS-located appetite regulatory centres due to their differential endocytic profiles, or the existence of *tissue-specific signalling* between GLP-1R-expressing neurons *versus* pancreatic β -cells. Ligand access to the brain and pancreas was directly assessed using fluorescently-tagged forms of the biased GLP-1RAs and was found to be similar between the agonists, thereby excluding the first of the two possibilities. In addition, these experiments suggested the brain uptake of exendin-4-based ligands to be independent of GLP-1R endocytosis at the blood-brain and blood-CSF barriers, challenging a recently proposed hypothesis in the literature.

Subsequent investigations comparing responses to the biased GLP-1RAs in neuronal and pancreatic β -cell lines revealed a different rank order of signalling efficacy between the two cell types, and pointed towards differences in the level of GLP-1R expression as an underlying cause. Thus, these data suggest the existence of tissue-specific signalling between the brain and the pancreas, and that this accounts for the divergent glycaemic and anorectic effects of exendin-4-based biased GLP-1RAs.

Finally, the *in vitro* and *in vivo* properties of pharmacokinetically optimised, acylated forms of the same biased GLP-1RAs were characterised. Interestingly, these studies demonstrated the glycaemic and anorectic efficacy of the biased GLP-1RAs to differ between lean mice and a mouse model of T2DM and obesity. Thus, in addition to tissue-specific signalling, pathophysiological changes in the diabetic, obese state appear to impact biased agonism at the GLP-1R.

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Declaration of Contributors

The work presented in this thesis was conducted primarily by the author. All collaboration and assistance received is described below.

Synthesis and purification of fluorescently-tagged peptides by click chemistry was conducted by Dr Maria Shchepinova, Department of Chemistry, Imperial College London.

HPLC analysis of DPP-4-mediated peptide degradation was conducted by Mr Zijian Fang.

Assistance with OPT imaging was provided by, and custom MATLAB scripts for OPT image analysis were written by Dr James McGinty, Photonics Group, Department of Physics, Imperial College London.

Lateral ventricle cannulations were carried out by Dr David Ma, Section of Investigative Medicine, Imperial College London, with assistance of the author.

Library preparation and DESeq2 analysis was performed by Miss Grazia Pizza and Dr Aida Martinez Sanchez, Section of Cell Biology and Functional Genomics, Imperial College London. Libraries were analysed in the Imperial BRC Genomics Facility.

Preparation of MIN6B1 cells for transcriptomic analysis was performed by Dr Stavroula Bitsi and Dr Alejandra Tomas, Section of Cell Biology and Functional Genomics, Imperial College London.

Glp1r-Cre/GCaMP3 mice were provided by Professor Stefan Trapp, University College London. Ca²⁺ imaging experiments were performed jointly with Miss Wanqing Jiang, University College London.

Assistance with tissue clearing and *in vivo* food intake and glucose tolerance experiments was provided by Miss Tanyel Ashik. Assistance with *in vivo* experiments was also provided by Mr Phil Pickford and Dr Ben Jones.

Acknowledgments

Firstly, I would like to express my immense gratitude to my supervisors. To Professor Sir Steve Bloom, for providing me with the opportunity to undertake this PhD, and for his seemingly limitless wisdom and witticisms. To Dr James Minnion, for supporting me throughout the final year of my PhD, and for always having my best interests at heart. To Dr Emilie Stolarczyk, for her care and camaraderie, and for her excellent teaching that laid the foundations for my development as a scientist. Above all, to Dr Ben Jones, for his mentorship and unwavering support, and for continually driving me to be a better scientist.

To members of the laboratory, for allowing me to leave my time at Imperial College with many fond memories. In particular, I am grateful for the friendship and support of P.P., M.N., I.F.F., Y.P., A.R., R.H., A.G., C.H., C.B., A.D., A.B., A.M., N.G.A., A.F.R., E-R.M., S.C., B.O., and T.A.

To my parents, for imparting upon me the value of education, and for your countless sacrifices to provide me with a wealth of opportunity. I dedicate this thesis to you. To my brothers, extended family, and friends, for your continual support throughout the years.

Finally, to L.J.H., for your love, care, support, and unfailing belief in me, without which I often wonder whether completing this PhD would have been possible.

Acronyms

α -MSH	α -melanocyte stimulating hormone
ABC	ATP-binding cassette
AC	Adenylyl cyclase
ACSF	Artificial cerebrospinal fluid
AD	Alzheimer's disease
ADP	Adenosine diphosphate
AgRP	Agouti-related peptide
Aib	α -aminoisobutyric acid
AMPA	AMPA receptor
AMPK	Adenosine monophosphate protein kinase
AMT	Adsorptive-mediated transcytosis
AP	Area postrema
ARC	Arcuate nucleus
AT1R	Angiotensin II type 1 receptor
ATP	Adenosine triphosphate
AUC	Area under the curve
BAD	Bcl-2-associated death promoter
BBB	Blood brain barrier
BCSFB	Blood CSF Barrier
BETP	4-(3-(benzyloxy)phenyl)-2-(ethylsulfinyl)-6-(trifluoro-methyl)pyrimidine
BG	Benzyl guanine
BMI	Body mass index
cAMP	Cyclic adenosine monophosphate
CART	Cocaine- and amphetamine-regulated transcript
CCK	Cholecystokinin
CGRP	Calcitonin-gene related peptide
CHB	Common hepatic branch
CHp	Choroid plexus
CMT	Carrier-mediated transport
CNS	Central nervous system
Cpa1	Carboxypeptidase A
CREB	cAMP response element-binding protein
CRH	Corticotropin-releasing hormone
CSF	Cerebrospinal fluid
CuAAC	Copper-catalysed alkyne–azide cycloaddition
CVO	Circumventricular organ
DERET	Diffusion-enhanced resonance energy transfer
DIO	Diet-induced obese
DMH	Dorsomedial hypothalamus
DMV	Dorsal motor nucleus of the vagus
DPP-4	Di-peptidyl peptidase-4

DVC	Dorsovaginal complex
EC	Endothelial cell
ECD	Extracellular domain
ECL	Extracellular loop
EGFP	Enhanced green fluorescent protein
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
Epac2	cAMP-regulated guanine nucleotide exchange factor 2
ERK	Extracellular signal-related kinase
ESC	Embryonic stem cell
FBS	Foetal bovine serum
Fc	Fragment crystallisable
FcRn	Neonatal Fc receptor
FDA	Food and Drug Administration
FFA	Free fatty acids
FFA1	Free fatty acid receptor 1
FGF21	Fibroblast growth factor 21
FITC	Fluorescein isothiocyanate
FR α	Folate receptor α
γ -Glu	γ -glutamic acid
GABA	γ -aminobutyric acid
GCGR	Glucagon receptor
GFP	Green fluorescent protein
GHSR	Growth hormone secretagogue receptor
GIP	Glucose-dependent insulinotropic polypeptide
GIPR	Glucose-dependent insulinotropic polypeptide receptor
GLP-1	Glucagon-like peptide-1
GLP-1R	Glucagon-like peptide-1 receptor
GLP-1RA	Glucagon-like peptide-1 receptor agonist
GLP-2	Glucagon-like peptide-2
GnRH	Gonadotrophin-releasing hormone
GPCR	G-protein coupled receptor
GRK	G-protein receptor kinase
GRPP	Glicentin-related pancreatic polypeptide
GTT	Glucose tolerance test
HP	Hippocampus
HPV	Hepatoportal vein
HTRF	Homogenous time resolved fluorescence
IBMX	3-isobutyl-1-methylxanthine
ICD	Intracellular domain
ICL	Intracellular loop
ICV	Intracerebroventricular
IPGTT	Intraperitoneal glucose tolerance test
iPSC	Induced pluripotent stem cell

IR	Insulin receptor
IV	Intravenous
LepR	Leptin receptor
LHA	Lateral hypothalamic area
IPBN	Lateral parabrachial nucleus
LRP	Low density lipoprotein (LDL)-receptor-related protein
LS	Lateral septum
MAPK	Mitogen-activated protein kinase
MC3R	Melanocortin-3 receptor
MC4R	Melanocortin-4 receptor
ME	Median eminence
MesNa	2-sulfanylethanesulfonate
MOR	μ -opioid receptor
MPGF	Major proglucagon fragment
NAc	Nucleus accumbens core
NEP	Neutral endopeptidase
NMR	Nuclear magnetic resonance
NPY	Neuropeptide Y
NTS	Nucleus of the solitary tract
OGTT	Oral glucose tolerance test
OPT	Optical projection tomography
OVLT	Organum vasculosum of the lamina terminalis/vascular organ of the lamina terminalis
OXM	Oxyntomodulin
p90RSK	Ribosomal s6 kinase
PC	Prohormone convertase
PD	Parkinson's disease
PECAM-1	Platelet endothelial cell adhesion molecule 1
PKA	Protein kinase A
POMC	Pro-opiomelanocortin
PPG	Preproglucagon
Pra	Propargylglycine
PVN	Paraventricular nucleus of the hypothalamus
PVp	Periventricular nucleus of the hypothalamus
PYY	Peptide YY
RME	Receptor-mediated endocytosis
RMT	Receptor-mediated transport/transcytosis
ROI	Region of interest
SCO	Subcommissural organ
sCy7	Sulfo-cyanine 7
SDA	Subdiaphragmatic vagal deafferentation
SFO	Subfornical organ
SLC	Solute carrier
SNP	Single nucleotide polymorphism
T2DM	Type 2 diabetes mellitus

TfR	Transferrin receptor
TMD	Transmembrane domain
TMDD	Target-mediated drug disposition
TR-FRET	Time-resolved Förster resonance energy transfer
TRF	Time resolved fluorescence
TU	Tuberal nucleus
vHP	Ventral hippocampus
vmARC	Ventromedial ARC
VMH	Ventromedial hypothalamus
VT750	VivoTag 750
VTA	Ventral tegmental area

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1 Introduction

1.1 Type 2 diabetes & obesity

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder in which blood glucose homeostasis is dysregulated due to impaired insulin secretion and tissue insulin resistance [1]. The resulting state of chronic hyperglycaemia (elevated blood glucose levels) predisposes to a range of secondary complications, including cardiovascular disease, blindness, kidney failure, and limb amputation. T2DM is a growing global and national health concern. Globally, its prevalence quadrupled between 1980 and 2014 [2]; in the UK, 3.8 million individuals suffer from T2DM, a figure projected to rise to 5.5 million by 2035. Presently in the UK, T2DM and its associated complications generate direct healthcare costs of £10 billion, and wider costs to the economy of £13 billion [3].

The increase in T2DM prevalence has been driven by parallel increases in obesity: a state of excessive adiposity arbitrarily defined as a body-mass index (BMI) of ≥ 30 . Obesity raises the risk of developing T2DM by 7-fold [4], and the frequent co-occurrence of the two diseases and their linked pathophysiological mechanisms has led to the coining of the term “diabesity”. Despite the pandemic proportions of these twin diseases, the majority of existing treatments for T2DM and obesity lack efficacy or are associated with undesirable side effects. Lifestyle interventions to promote weight loss (namely, dieting and physical exercise) have poor long-term adherence and limited effectiveness [5]–[7]. Classical T2DM treatments of metformin, sulfonylureas and insulin treatment are associated, respectively, with inadequate glycaemic control, hypoglycaemic episodes, and weight gain [1], [8]. Orlistat, the sole UK licensed anti-obesity pharmacotherapy until 2017, causes only modest weight loss and unpleasant gastrointestinal side effects [9]. Thus, there is a pressing need for new treatments.

In contrast, bariatric surgery is a highly effective therapy for both T2DM and obesity. It leads to substantial and sustained weight loss, and long-term remission of T2DM [10], [11]. However, the risk of perioperative death and postoperative complications (including postprandial hypoglycaemia and nutrient deficiencies) [12]–[15] mean that it is typically reserved for individuals who are morbidly obese (BMI ≥ 40 , or BMI of 35–40 with an associated obesity-related health condition) [16]. It is therefore not a tenable universal treatment.

One of the mechanisms by which bariatric surgery leads to therapeutic efficacy is the enhanced postprandial secretion of gut-derived peptides, including glucagon-like peptide 1 (GLP-1) [17]. GLP-1 has potent insulinotropic and appetite-suppressive effects and thus pharmacotherapies that mimic GLP-1's physiological action have been developed for T2DM. This class of drugs represents an attractive treatment option as, in addition to achieving glycaemic control, their anorectic effects promote weight loss and thereby lead to broader metabolic improvements. Additionally, more recent members of this drug class with optimised weight-lowering properties are now licensed for the treatment of obesity, even in the absence of T2DM. An investigation into how an exemplar of this drug class mediates its physiological effects is the focus of this thesis.

1.2 Normal glucose homeostasis

In healthy humans, blood glucose levels are tightly regulated between 4 and 7 mmol/L despite large daily fluctuations in the rate of glucose delivery to, and removal from the circulation (e.g. after meal ingestion and during exercise, respectively). Such control is achieved by a coordinated hormonal and neural system involving the pancreas, brain, liver, intestine, skeletal muscle, and adipose tissue, and is critical in light of the multifarious pathologies associated with both hypo- and hyperglycaemia. Glucose is a critical fuel for all tissues; in particular, the brain is an obligate glucose consumer and will only utilise other metabolic substrates (e.g. ketone bodies) after prolonged fasting. The two major regulators of glucose homeostasis are insulin and glucagon; two pancreatic hormones that have broadly opposing functions.

1.2.1 Insulin

The primary glucoregulatory hormone is insulin: a peptide hormone produced by the β -cells of the islets of Langerhans in the pancreas [18]. Insulin acts to stimulate glucose uptake by insulin-sensitive tissues (in particular skeletal muscle and adipose tissue), induce the conversion of excess glucose to glycogen in the liver, and inhibit the hepatic glucose-producing processes of glycogenolysis (the breakdown of glycogen to liberate glucose) and gluconeogenesis (the *de novo* synthesis of glucose from non-carbohydrate precursors, including amino acids and lactate). Insulin secretion occurs in response to meal ingestion, and may occur after direct sensing of glucose and amino acids by β -cells [19], or in response to intestinally-secreted insulinotropic peptides (as discussed below).

1.2.2 Glucagon

Glucagon is the major counter-regulatory hormone to insulin, and is produced by α -cells of the islets of Langerhans [18]. Glucagon regulates hepatic glucose production by inducing glycogenolysis and gluconeogenesis, and inhibiting glycolysis (the breakdown of glucose). It therefore raises plasma glucose in fasting conditions by increasing hepatic glucose output. Glucagon secretion is triggered by the detection of declining blood glucose levels by α -cells, or by elevated blood levels of amino acids (e.g. after a protein-rich meal) [20], [21]; conversely, its secretion is inhibited by β -cell-released insulin, along with other intra- and extra-pancreatic factors [22].

1.2.3 Incretin hormones

Circulating nutrients can directly stimulate insulin release from pancreatic β -cells. In addition, peptide hormones secreted from the intestine postprandially – termed “incretins” – also serve to modulate insulin secretion. The notion that substances produced by the intestinal mucosa stimulated the secretion of glucose-lowering factors by the endocrine pancreas was first noted in the early 1900s [23]. However, the physiological relevance of this gut-pancreas communication was only demonstrated in the 1960s, when it was shown that oral glucose administration induced significantly greater insulin secretion when compared to an equivalent glucose dose given intravenously [24]. This phenomenon was termed the “incretin effect”, and is estimated to account for 50-70% of insulin secreted after oral glucose administration [25].

Two peptide hormones are responsible for this effect, glucose-dependent insulintropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), which are released from intestinal K-cells and L-cells, respectively, and bind to cognate receptors the GIP receptor (GIPR) and GLP-1 receptor (GLP-1R) [26]. The relative importance of GIP and GLP-1 in the incretin effect has been contested since their discovery. Whilst GIP circulates at higher concentrations than GLP-1 [27], pancreatic β -cells respond to lower concentrations of GLP-1 than GIP [28]. Historical studies in humans infusing the peptides at their respective physiological concentrations have variably reported a dominant role for GLP-1 and GIP [29], [30]. Whilst the existence of a potent and specific GLP-1R antagonist, exendin(9-39) [31], has previously allowed investigation of GLP-1's contribution to the incretin effect in humans [32], only recently has an equivalently effective GIPR antagonist, GIP(3-30)NH₂ been discovered [33]. Utilising these two antagonists, a recent study in healthy humans concluded that GIP plays a greater role in postprandial insulin secretion and glucose homeostasis, although both hormones contribute additively under normal conditions [34]. By contrast, in T2DM patients, GIP's incretin effects are lost irrespective of the dose administered [35], [36], whereas supraphysiological doses of GLP-1 effectively correct hyperglycaemia [35].

1.3 Pathophysiology of T2DM

In contrast to the tight regulation of blood glucose in healthy individuals, glucose homeostasis is dysregulated in T2DM. The core metabolic defects in T2D result from impaired insulin secretion by pancreatic β -cells and insulin resistance in peripheral tissues [1]. The consequences of the resulting state of hyperglycaemia are manifold: macrovascular complications include stroke and coronary heart disease, and microvascular complications include retinopathy, nephropathy and neuropathy. Diagnostically, T2DM is defined by the possession of any one of the following criteria [37]:

1. HbA1c $\geq 6.5\%$
2. Fasting plasma glucose ≥ 7.0 mmol/L
3. Plasma glucose ≥ 11.1 mmol/L two hours after a 75 g oral glucose tolerance test
4. Classical symptoms of hyperglycaemia accompanied by a random plasma glucose of ≥ 11.1 mmol/L

Both genetic and environmental factors contribute to the aetiology of T2DM. Studies in monozygotic twins suggest that 72% of T2DM susceptibility is heritable [38]. Genome wide association studies have identified numerous common variants associated with T2DM [39], [40]. Most of these are in intronic, but some are located in exons of genes related to glucose homeostasis, including *GCKR* (encoding glucokinase regulatory protein), *TCF7L2* (a transcription factor regulating the expression of genes involved in insulin granule exocytosis) and *SLC30A8* (encoding a zinc transporter involved in insulin storage) [39], [41], [42]. However, even in aggregate, all identified variants only account for ~10% of overall T2DM heritability [39], [40]. The genetic architecture of T2DM therefore appears to one with minor contributions from multiple loci, which cumulatively confer disease susceptibility. Environmental changes promoting increased caloric intake and decreased energy expenditure have provided the ideal conditions for disease development in genetically predisposed individuals, thereby precipitating the T2DM pandemic.

The pathophysiology of T2DM can be broadly summarised by the following events [1]:

1. β -cell dysfunction: β -cells incur glucotoxic, lipotoxic and cytotoxic damage due to the raised circulating levels of glucose [43], free fatty acids (FFA) [44], [45], and pro-inflammatory cytokines [46], respectively, that are associated with states of obesity and insulin resistance. These systemic insults impair insulin secretion and lead to β -cell apoptosis. Additionally, increased demand for insulin protein synthesis (given resistance of insulin-responsive peripheral tissues) [47], [48] and amyloid deposition [49] cause endoplasmic reticular and oxidative cellular stress, thereby exacerbating β -cell dysfunction and death.
2. Raised hepatic glucose production: raised fasting glucagon levels, failure to suppress glucagon levels postprandially, liver insulin resistance, and increased hepatic sensitivity to glucagon all contribute to a state of chronic hyperglycaemia [50]–[56].
3. Insulin resistance in skeletal muscle: leads to reduced glucose uptake, thereby contributing to hyperglycaemia [56].
4. Insulin resistance in adipose tissue: leads to reduced glucose uptake, but also increased lipolysis (due to the loss of insulin's anti-lipolytic effect) and raised FFA secretion into the blood [51]. This, in turn, aggravates β -cell dysfunction and contributes to muscle and liver insulin resistance.
5. Increased renal re-absorption of glucose and kidney insulin resistance: both of these events contribute to chronic hyperglycaemia [57].
6. β -cell insensitivity to GLP-1 and GIP: loss of responsiveness to the incretin hormones leads to insufficient insulin secretion, and negates the anti-apoptotic effects of these hormones upon β -cells [35].
7. Neuronal resistance to regulators of energy homeostasis: resistance to insulin, leptin, GLP-1, and peptide YY diminish the chronic and acute appetite-suppressive effects of these hormones, promoting weight gain and an insulin resistant state [1].

The pathophysiology of T2DM is therefore complex and self-propagating due to the existence of positive feedback between the described disease events. As such, overt T2DM is typically preceded by “prediabetes”: an intermediate state of hyperglycemia, where glycaemic parameters are above a healthy range but below the threshold for T2DM diagnosis (i.e. HbA1c of 5.7-6.4%) [1], [37]. Screening for the prediabetic state is thus recommended to provide opportunities to intervene prior to the onset of T2DM proper [58], [59].

1.4 The homeostatic regulation of food intake

As with blood glucose, food intake is under careful homeostatic regulation. Within the brain, the hypothalamus and hindbrain contain numerous neurocircuits critical to the control of food intake [60], [61]. Both structures lie in close proximity to circumventricular organs (CVOs; regions of the brain with a “leaky” blood brain barrier due to the presence of fenestrated capillaries) and are therefore capable of directly sensing peripherally-derived, circulating signals indicative of the body's acute nutritional status and longer-term energy balance. The hindbrain also receives neural input from vagus nerve afferents that relay neuropeptidergic and mechanical satiety signals from the gastrointestinal tract. Extensive reciprocal connections exist between the hypothalamus and hindbrain to integrate activity between the two regions and to thereby achieve the coordinated control of energy homeostasis.

1.4.1 Hypothalamic control of food intake

The arcuate nucleus (ARC) is perhaps the most intensively studied of the hypothalamic nuclei involved in energy homeostasis. The ARC contains two populations of antagonistic neurons: the anorexigenic pro-opiomelanocortin (POMC)- and cocaine- and amphetamine-regulated transcript (CART)-expressing neurons, and orexigenic neuropeptide Y (NPY)- and agouti-related peptide (AgRP)-expressing neurons [62], [63]. Under conditions of positive energy balance, POMC/CART neurons release neuropeptide α -melanocyte stimulating hormone (α -MSH) that activates melanocortin-3 and -4 receptors (MC3/4Rs) on second-order neurons in the paraventricular nucleus (PVN), dorsomedial hypothalamus (DMH), lateral hypothalamic area (LHA), and ventromedial hypothalamus (VMH) [61], [64], [65]. Signalling within these regions, and to extrahypothalamic areas, leads to the inhibition of food intake and an increase in energy expenditure.

By contrast, NPY/AgRP neurons are activated in conditions of negative energy balance and weight loss, and release neuropeptides NPY and AgRP [62]. NPY directly stimulates food intake and decreases energy expenditure by activating NPY Y1 and Y5 receptors expressed in the PVN and LHA [66]–[68], whilst AgRP indirectly inhibits food intake by acting as an inverse agonist of MC3/4Rs [69]. Additionally, NPY/AgRP neurons directly inhibit POMC/CART neurons by γ -aminobutyric acid (GABA) signalling [70]. Interestingly, optogenetic and chemogenetic studies report that whilst activation of NPY/AgRP neurons results in the immediate onset of feeding, POMC/CART neurons require chronic stimulation in order to inhibit appetite [71]–[73]. Such findings suggest that POMC/CART neurons play a greater role in long-term energy balance, whereas AgRP neurons are capable of mediating acute responses. Recently, another population of glutamatergic ARC neurons that rapidly inhibit appetite have been identified; these neurons project to MC4R-expressing PVN neurons, and transmission in this neurocircuit is potentiated by α -MSH [74]. Thus, unlike POMC/CART neurons, this glutamatergic ARC-PVN neuronal population may act at an equivalent timescale to NPY/AgRP neurons to antagonise their orexigenic effects.

The ARC is adjacent to circumventricular organ the median eminence (ME) and is therefore in a position of relative accessibility to blood-borne signals. Leptin and insulin are two major signals indicative of overall energy balance that are integrated by hypothalamus. Leptin is an adipocyte-produced hormone that circulates at levels proportional to body-fat mass [75] and enters the brain at levels proportional to plasma concentration [76]. Leptin activates leptin receptors (LepRs) expressed by POMC/CART and NPY/AgRP neurons to cause neuronal activation and inhibition, respectively [70]. Likewise, insulin circulates in proportion to overall adiposity [77], and serves to activate POMC/CART, and inhibit NPY/AgRP neurons via the insulin receptor (IR) [78], [79]. In addition to these longer-term signals of energy balance, ARC neuronal populations are responsive to short-term satiety signals. NPY/AgRP neurons express receptors for and are activated *in vivo* by ghrelin, an orexigenic peptide predominantly produced by P/D₁ cells of the stomach in response to fasting [80], [81]. Both POMC/CART and NPY/AgRP neurons express the Y₂ receptor for, and are responsive to peptide YY (PYY), an anorectic peptide produced by L cells of the intestine in response to meal ingestion [82]. Finally, a small proportion of POMC/CART neurons express the GLP-1R [83] although the direct action of peripherally-derived GLP-1 upon these neurons is disputed due to its short circulating half-life (as discussed below).

1.4.2 Hindbrain control of food intake

Within the hindbrain, the critical neural substrates involved in the regulation of food intake are the nucleus of the solitary tract (NTS), area postrema (AP), dorsal motor nucleus of the vagus (DMV), and lateral parabrachial nucleus (IPBN). The former three nuclei collectively comprise the dorsal vagal complex (DVC). Vagal afferent neurons terminate within the NTS and thus the NTS integrates gastrointestinal satiety signals, namely gastric distension [84] and gut-derived neuropeptides [85]. Vagal afferents express receptors for GLP-1, PYY, ghrelin, and anorectic cholecystokinin (CCK) and, as demonstrated by vagal deafferentation or total vagotomy, appear to mediate the anorectic effects of these peptides, at least in part [86]–[90].

Additionally, NTS neurons themselves express receptors for GLP-1 [91], PYY [92], CCK [93], POMC [64], leptin [94], and ghrelin [95], and are activated by peripheral injection of these peptides [96]–[100]. The NTS also possesses subpopulations of neurons that produce POMC and GLP-1, and whilst they appear to be dispensable for the long-term control of appetite and body weight regulation, these subpopulations mediate distinct aspects of feeding behaviour [73], [101], [102]. The NTS integrates descending input from many appetite-regulating neuronal populations, including the ARC POMC/CART neurons [103], orexigenic orexin- and melanocortin hormone-expressing neurons of the LHA [104], [105], and NPY-expressing neurons of the DMH [106]. The NTS, in turn, signals in to the IPBN [107] and PVN [108] in distinct appetite-regulating neurocircuits.

The AP expresses receptors for GLP-1 [91], CCK [93], ghrelin [109], leptin [94], and PYY [92] and, as a circumventricular organ, is capable of directly sensing systemic factors. Interestingly, ablation of the AP attenuates the effects of CCK [110], ghrelin [109] and leptin [111], but is not necessary for those of PYY [112] or GLP-1R agonists [113]. The AP forms reciprocal projections with the NTS and IPBN, and receives input from the PVN and DMH [114].

Finally, and in contrast to the AP and NTS, the DMV does not have a sensory role. It contains the cell bodies of parasympathetic motor vagal efferent neurons, and acts as an effector nucleus to control gastric and pancreatic function [115]. Its activity is modulated by the NTS, from which it receives dense innervation [115]. Thus, the DVC nuclei make key contributions to appetite regulation by: a) directly sensing circulating appetite-regulating hormones; b) receiving ascending vagal signals from the gastrointestinal tract; c) integrating descending input from hypothalamic and extrahypothalamic areas; and d) effecting behavioural change via descending vagal fibres.

The DVC has long been recognised as important to appetite regulation; more recently, the IPBN has emerged as a pivotal relay centre in the control of food intake. Calcitonin-gene related peptide (CGRP)-expressing neurons within this region are activated by physiological satiety signals including gastric distension, CCK, and GLP-1, as well as aversive stimuli (nausea-inducing lithium chloride and lipopolysaccharide). Experimental activation of CGRP neurons causes rapid, profound anorexia that, if sustained, induces substantial weight loss [107], [116]. Conversely, their inactivation abolishes the anorectic effects of CCK, GLP-1 and leptin, and increases meal size; however, net food intake is unchanged due to a corresponding reduction in meal number [107]. Thus, and in direct contrast to ARC POMC/CART neurons, PBN CGRP neurons provide an important meal termination signal but do not appear to be required for long-term energy homeostasis. In keeping with this phenotype, CGRP neurons receive inhibitory descending GABAergic input from AgRP neurons of the ARC [117], and

excitatory glutamatergic ascending input from the NTS [118]. An NTS-IPBN-ARC neurocircuit has therefore emerged as critical to the physiological control of appetite, facilitating signal integration between the hypothalamus and brainstem [119].

1.5 GLP-1 production

Given that the ingestion of a mixed meal supplies glucose, it is perhaps unsurprising that shared regulators of glucose homeostasis and food intake, such as GLP-1, exist. Before discussion of its therapeutic application, its endogenous production, degradation, and effects will be considered.

GLP-1 is encoded by the preproglucagon gene (*Gcg*) that is expressed in α -cells of the pancreas, L-cells of intestine, and specific neuronal populations in the central nervous system (CNS) [120], [121]. The preproglucagon gene product is post-translationally cleaved by prohormone convertases (PCs) in a tissue specific-manner, resulting in different peptide products [122] (Figure 1.1). In α -cells, expression of PC2 produces glucagon, glicentin-related pancreatic polypeptide (GRPP) and the major proglucagon fragment (MPGF). In L-cells and neurons, expression of PC isoform PC1/3 generates GLP-1, glucagon-like peptide 2 (GLP-2), and glicentin, the latter of which may be further cleaved into oxyntomodulin and GRPP.

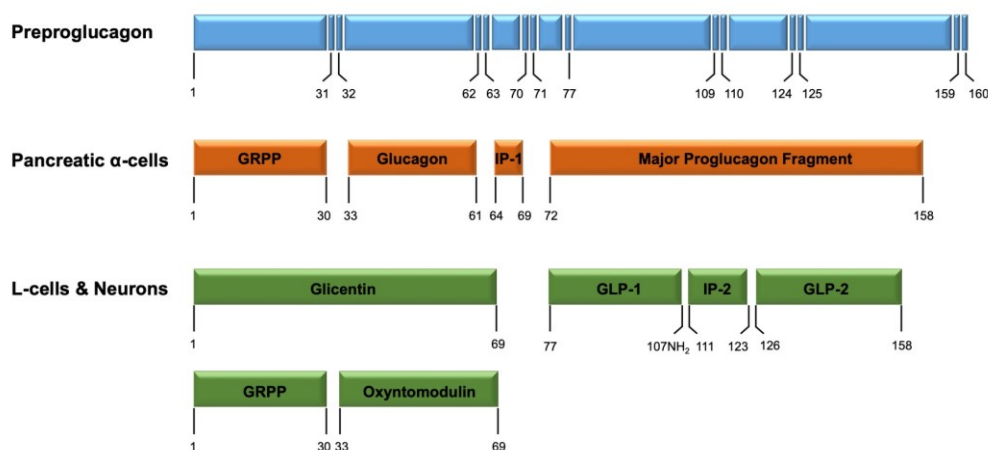


Figure 1.1. Post-translational processing of preproglucagon.

Differential post-translational processing of the preproglucagon in pancreatic α -cells, L-cells and neurons. GRPP: glicentin-related pancreatic polypeptide. GLP-1: Glucagon-like peptide-1. GLP-2: Glucagon-like peptide-2. IP-1: Intervening peptide-1. IP-2: Intervening peptide-2.

Full length GLP-1 - GLP-1(1-37) - comprises 37 amino acids, but is cleaved and C-terminally amidated to generate the primary biologically active form of GLP-1, GLP-1(7-36)NH₂ (sometimes denoted GLP-1(7-37)) [123]. GLP-1 is secreted by L-cells in response to nutrient ingestion in a biphasic pattern, with a first phase occurring within 10 to 15 minutes of meal ingestion, and a second phase within 30 to 60 minutes [124]. L-cells are present along much of the length of the intestine, beginning at the proximal jejunum and increasing in density along the ileum and colon [125]. L-cells are capable of directly sensing intraluminal nutrients via specific transporters and receptors, including glucose via the sodium-coupled glucose transporter 1 (SGLT1) [126], amino acids via the calcium sensing receptor [127] and GPR142 [128], and fatty acids via the free fatty acid receptor 1 (FFA1 or Gpr40) [129]. As L-cells are located in the distal intestine, it is unlikely that direct nutrient sensing

accounts for the early phase of GLP-1 secretion, and evidence exists for a vagally-mediated mechanism mediating this component of the L-cell response [130].

1.5.1 GLP-1 degradation

GLP-1 is rapidly degraded by di-peptidyl peptidase-4 (DPP-4) and neutral endopeptidase NEP-24.11, resulting in a circulating half-life of 1-2 minutes [131]–[133]. DPP-4 is a serine protease that cleaves peptides between amino acids 2 and 3, particularly if the second amino acid is an alanine or proline [134]. GLP-1 possesses an alanine as its penultimate residue, and is therefore cleaved to the biologically inactive form GLP-1(9-36)NH₂. NEP-24.11 further cleaves GLP-1 at six sites in the central and C-terminus peptide regions (Asp15-Val16, Ser18-Tyr19, Tyr19-Leu20, Glu27-Phe28, Phe28-Ile29 and Trp31-Leu32) [132].

GLP-1 degradation after L-cell secretion is extensive. DPP-4 is expressed at high levels on gut capillary endothelial cells that lie adjacent to L-cells [135], and it is estimated that less than 25% of secreted GLP-1 reaches the hepatic portal circulation in intact form [136]. Further degradation in the liver diminishes systemically circulating GLP-1 to only 10-15% of that originally produced [137], [138]. Remaining active GLP-1 is degraded by the soluble form of DPP-4 present in the circulation, and membrane-bound DPP-4 expressed in various other tissues including the kidney, spleen, pancreas and brain [139]. Elimination of inactive GLP-1(9-36)NH₂ occurs primarily in the kidney, resulting in a half-life of 4-5 minutes [140].

1.6 Physiological actions of GLP-1

The GLP-1R is widely expressed, including in the pancreatic islets, brain, vagus nerve, liver, heart, kidney, and gastrointestinal tract [141], [142]. Correspondingly, the actions of GLP-1 are diverse. Its metabolic effects include the stimulation of insulin secretion [29], inhibition of glucagon secretion [143], exertion of anti-apoptotic and trophic effects upon β -cells [144], [145], inhibition of gastric emptying (“ileal brake effect”) [146], and suppression of food intake [147]. Additionally, GLP-1 confers neuroprotective [148] and cardioprotective effects [149], coordinates bone formation and resorption [150], and modulates kidney natriuresis [151]. Given the focus of this thesis, only the incretin and anorectic effects of GLP-1 will be reviewed in detail here.

1.6.1 Incretin effects of GLP-1

GLP-1 is a potent insulin secretagogue. Intravenous infusion of GLP-1 greatly enhances insulin release and corrects hyperglycaemia in both normal humans [29] and type 2 diabetic patients [35]. Supporting a physiological role in humans, GLP-1 exhibits a post-prandial release pattern that correlates with insulin secretion [152], and infusion of selective GLP-1R antagonist exendin(9-39) worsens postprandial glycaemic control [32]. Notably, the preservation of GLP-1’s insulinotropic effects in diabetic patients has led to the development of pharmacological GLP-1R agonists (GLP-1RAs) as anti-diabetic medications. This approach has been highly successful, as discussed below. Despite its well-established insulinotropic action, how GLP-1 exerts this effect is still the subject of controversy.

1.6.1.1 Endocrine mechanism

The GLP-1R is expressed at high levels in pancreatic β -cells [142] and thus endogenously produced GLP-1 may act directly on the pancreas via the systemic circulation to stimulate insulin secretion. Modulation of insulin release by intravenous infusion of GLP-1 at physiological levels, or using the GLP-1R antagonist exendin(9-39), constitute the traditional evidence for this notion [29], [32], [153]. However, the short circulatory half-life of GLP-1 casts doubt upon the feasibility of a primarily endocrine mode of action. In humans in the postprandial state, peak levels of active GLP-1(7-36)NH₂ are less than 10 pM [154]. This may still be sufficient for GLP-1R activation, as Ca²⁺ responses were observable in *ex vivo* human pancreatic islets with GLP-1 stimulation in the femtomolar range [155]. Admittedly though, the translation of *in vitro* pharmacology to an *in vivo* system is problematic. In further support for the classical incretin model, the selective re-introduction of GLP-1R expression in β -cells of global *Glp1r*^{-/-} mice was sufficient to normalise abnormal glucose homeostasis [156].

1.6.1.2 Neural mechanism

In light of low concentrations of GLP-1 reaching β -cells due to rapid degradation, it has been proposed that L-cell-released GLP-1 instead acts locally upon the vagus nerve to engage a gut-brain-islet circuit mediating insulin secretion. An anatomical substrate for this exists: vagal afferent terminals lie in close proximity to L-cells [157], express the GLP-1R [158], signal to central nuclei regulating energy homeostasis [159], [160], and have efferent projections to islets [161]. Importantly, strong functional evidence for this circuit exists. GLP-1R knockdown in vagal afferents by lentiviral injection into the nodose ganglion (where vagal afferent cell bodies reside) reduced postprandial insulin secretion and worsened glucose handling [162]. Likewise, subdiaphragmatic vagal deafferentation (SDA) in mice exacerbated glucose handling after an oral glucose load [86]. Notably, administration of exendin(9-39) did not further exacerbate the glucose excursion, suggesting that endogenous GLP-1 primarily mediates its glucose-lowering effects in a vagal-dependent manner [86]. Moreover, a very recent study demonstrated that GLP-1R deletion from *Phox2b*-expressing neurons (including vagal afferents in the nodose ganglion, neurons of the NTS to which vagal afferents project, and vagal efferents projecting from the DMV) resulted in raised fasting levels of glucose, plasma insulin, glucagon and GLP-1, and significantly impaired responses to glucose challenge [163]. Finally, and in further support of a crucial role for extra-islet GLP-1Rs, mice with β -cell-specific knockout or inducible knockdown of the GLP-1R showed normal oral glucose responses [164]. These findings are, however, in direct contrast to those above demonstrating the primary importance of β -cell GLP-1Rs [156].

In addition to vagal afferents innervating the gut, GLP-1R-expressing vagal afferents terminate in the hepatoportal bed (common hepatic branch (CHB) of the vagus), providing another location for L-cell-secreted GLP-1 to potentially activate the vagus nerve. However, a role for the CHB in GLP-1-stimulated insulin secretion is contested. In support, hepatoportal vein (HPV) injections of low-dose GLP-1 [165] or DPP-4 inhibitors [166] caused enhanced glucose-stimulated insulin secretion that could be abolished by vagotomy. However, selective vagotomy of the CHB had no effect on glucose handling [86].

1.6.1.3 Paracrine mechanism

A further pancreatic α -cell-mediated mechanism for endogenous GLP-1's insulinotropic effects has been proposed. α -cells are traditionally known for their role in glucagon production, though evidence exists for their expression of PC1/3 and corresponding production of GLP-1 under pathophysiological conditions in both rodents [167]–[171] and humans [172]. These data suggest that α -cell-secreted GLP-1 is capable of acting in a paracrine manner to stimulate GLP-1Rs on β -cells, at least under conditions of metabolic stress. The appropriate cytoarchitecture exists for such action in humans [173], [174], and paracrine action of α -cell secreted acetylcholine upon β -cells has been described [175]. More recently, animal models support the importance of α -cell-secreted GLP-1 under physiological conditions. Intriguingly, using L-cell- or α -cell-specific restoration of preproglucagon gene (*Gcg*) expression in global *Gcg*^{-/-} knockout mice, Chambers et al. report α -cell but not L-cell GLP-1 expression to be fundamental to glucose metabolism [176]. However, other studies have failed to detect active GLP-1 in normal mouse islets [177], [178].

Thus, whether endogenously secreted GLP-1 enacts its insulinotropic effects in an endocrine, neural or paracrine manner is still contested, with a conservative interpretation being that all three mechanisms are relevant. However, the relative contribution of each under physiological and pathophysiological conditions remains to be clearly elucidated.

1.6.1.4 Incretin action of pharmacological GLP-1RAs and GLP-1 post bariatric surgery

Despite conflicting findings about the endocrine action of endogenous GLP-1, there is little doubt that pharmacological GLP-1RAs – with significantly extended half-lives – act directly upon β -cell GLP-1Rs. In mice with β -cell-specific knockdown of the GLP-1R, the anti-hyperglycaemic effects of a GLP-1RA liraglutide were impaired [164]. Consistently, the anti-hyperglycaemic effects of GLP-1RAs liraglutide and exendin-4 were restored in *Glp1r*^{-/-} mice with β -cell-specific rescue of GLP-1R expression [156]. In fact, β -cell GLP-1R expression appears to be sufficient for the glucoregulatory effects of pharmacological GLP-1RAs, as responses to liraglutide were unaffected in vagotomised mice [179] or mice with vagal nerve-specific deletion of the GLP-1R [163], [180].

Finally, another setting in which GLP-1 may act in an endocrine manner is post bariatric surgery. Here, levels of GLP-1 may be significantly elevated relative to unoperated control subjects [181], [182], and thus sufficient levels of unmetabolised GLP-1 may reach the pancreas. In support of this, β -cell-specific knockdown of the GLP-1R post vertical sleeve gastrectomy blunted surgery-induced glucoregulatory improvements in mice [183]. Thus, in conditions with high circulating levels of endogenous GLP-1 or pharmacological GLP-1RAs, direct agonism of β -cell GLP-1Rs may be the dominant mechanism for glucose-stimulated insulin secretion.

1.6.2 Anorectic effects of GLP-1

It has been robustly demonstrated that GLP-1 or GLP-1RA administration by intraperitoneal [184], intravenous [185], or central routes [186] leads to a reduction in food intake. However, reaching a clear understanding of how GLP-1 mediates its anorectic effects has been complicated by GLP-1's short half-life, the expression of the GLP-1R in vagal afferents as well as in numerous, distributed brain regions implicated in energy homeostasis, and the production of endogenous GLP-1 in the

brain. As with GLP-1's incretin effects, a key debate is whether the anorectic effects of L-cell secreted GLP-1 are mediated by local action on intestinal vagal afferents, or in an endocrine manner upon GLP-1Rs expressed in CNS-located appetite regulating centres. Overall, studies to date suggest a vagal mode of action for L-cell secreted GLP-1, though endocrine action for pharmacological GLP-1RAs.

1.6.2.1 *Peripheral anorectic action of GLP-1 & GLP-1RAs*

A number of lines of evidence suggest the importance of peripheral GLP-1Rs in GLP-1's anorectic effects. Firstly, the anorectic effects of peripherally administered GLP-1 were inhibited by peripheral but not central administration of exendin(9-39) [187]. Secondly, high-molecular weight and therefore brain-impermeant GLP-1-albumin and exendin-4-albumin conjugates still exerted anorectic effects after peripheral administration, and led to the expression of neuronal activation marker c-fos in central brain nuclei [188], [189]. Thirdly – and supporting the importance of vagal GLP-1Rs - vagotomy or capsaicin administration (a neurotoxin to lesion and render sensory nerves unresponsive) attenuated the anorectic effects of intraperitoneally administered GLP-1 in rats [87] and exendin-4 in mice [190].

The exact route of peripheral administration is important, as SDA [191] or complete vagotomy [192] were able to abolish the anorectic effects of intraperitoneal but not intravenous GLP-1 administration (into the hepatic portal vein, vena cava, or tail vein). It is therefore likely that intravenous infusions of GLP-1 achieve sufficiently high circulating concentrations for direct action upon central GLP-1Rs. By contrast, intraperitoneal administration may better mimic the situation for endogenously secreted GLP-1, where action is directly upon intestinal vagal afferents. Of note, the anorectic effects of intraperitoneally administered GLP-1 were maintained after specific lesioning of the CHB of the vagus but were abolished after complete subdiaphragmatic deafferentation, emphasising the criticality of intestinally located vagal afferents to GLP-1's anorectic effects [86].

1.6.2.2 *Central anorectic action of GLP-1 & GLP-1RAs*

Although it is unlikely that L-cell-secreted GLP-1 reaches the brain at physiologically relevant concentrations, GLP-1 produced locally in the brain is likely able to activate the extensive network of GLP-1R-expressing neurons in the CNS [193]. Crucially, the predominant source of brain GLP-1 is the NTS in the brainstem [101], to which vagal afferents project [159]. Thus, a working model for endogenous GLP-1's anorectic effects is as follows: L-cell-secreted GLP-1 activates vagal GLP-1Rs, leading to vagal signalling and secondary activation of NTS GLP-1-producing neurons; GLP-1 released by NTS neurons activates GLP-1R-expressing neuronal networks in other brain regions, leading to coordinated activity to suppress appetite.

In contrast to endogenous GLP-1, there is little doubt that supraphysiological levels of GLP-1 or pharmacological GLP-1RAs have direct effects upon central GLP-1Rs. Peripherally infused, iodinated GLP-1 achieved entry to the brain via circumventricular organs (CVOs) [152] and the blood brain barrier (BBB) [194] and showed binding in GLP-1R-expressing, feeding-related brain nuclei [195]. Moreover, the anorectic effects and body weight reductions associated with long-acting GLP-1RAs liraglutide and exendin-4 have been shown to involve direct action on central GLP-1Rs after blood brain barrier penetration [163], [179], [180], [196].

Irrespective of whether activation is direct or vagally-mediated, central neural circuits within the hindbrain, hypothalamus and cortex are critical to physiological and pharmacological GLP-1R-mediated anorectic effects. However, generating a coherent picture of GLP-1 action in the brain from the wealth of often region-specific data is an ongoing challenge.

1.6.2.2.1 *Hindbrain substrates*

Within the caudal brainstem, the NTS, AP and DVC express the GLP-1R [91], [197]. Intracerebral injection of exendin(9-39) into the medial NTS results in increased food intake, evidencing a physiological role for this neuronal population in GLP-1-induced anorexia [198]. Moreover, as demonstrated in supracollicular decerebrate rats, caudal brainstem circuits are sufficient to enact the acute anorectic effects of peripherally administered exendin-4 [199]. It should be acknowledged that although these data provide support for the sufficiency of the caudal brainstem in food intake control, the control of feeding behaviour in normal animals involves powerful descending input from, and integration of neural activity with other midbrain and forebrain regions discussed below [85]. Of the DVC nuclei, studies to date suggest that the NTS is the most important with regard to GLP-1-mediated anorexia [193].

In addition to housing neuronal populations responsive to systemic GLP-1RAs, a subpopulation of GLP-1R-negative [200], [201], preproglucagon-expressing NTS neurons (“PPG neurons”) produce GLP-1 [121], [202]. A number of lines of evidence support a crucial role for these neurons in the physiological control of appetite. Firstly, RNA interference (RNAi)-mediated knockdown of the preproglucagon gene in NTS results in hyperphagia and weight gain [203]. Secondly, PPG neurons innervate all brain regions identified as mediating GLP-1 effects [204]–[206], and are sensitive to peripheral satiety signals [200], [207], [208]. Finally, ablation of PPG neurons resulted in a significant reduction in GLP-1 levels in the hypothalamus and brainstem, supporting their status as the main source of central endogenous GLP-1 [101]. Surprisingly, a recent study suggested that these neurons are not involved in the primary control of food intake, but rather mediate a “secondary satiety signal” in response to psychogenic stress and large meals [101]. Holt et al. found ablation or chemogenetic inhibition of PPG neurons to have no effect upon *ad libitum* food intake or body weight, in direct contrast to the aforementioned RNAi studies [101], [203]. Thus, despite recognition of their importance, the precise role of NTS PPG neurons in the control of food intake is yet to be determined. Of particular note, the findings of Holt et al. challenge the prevailing hypothesis for the action of L-cell-secreted GLP-1 described above, thereby providing indirect support for an endocrine mode of action for peripherally released GLP-1. Confirmation of their findings is therefore of particular importance.

1.6.2.2.2 *Hypothalamic substrates*

The GLP-1R is expressed in most hypothalamic nuclei, including well-established appetite regulatory centres the ARC, PVN, VMH and LHA [91], [197]. Moreover, many of these regions receive direct innervation from, and are supplied with endogenous GLP-1 by PPG NTS neurons [101], [204]. Despite general recognition of their importance in physiological or pharmacological GLP-1R-mediated hypophagia, uncertainty exists as to the relative contributions of the various hypothalamic nuclei.

Sandoval et al. reported a functional dichotomy between the metabolic roles of the ARC and PVN, with intracerebral injection of GLP-1 into the ARC affecting glucose metabolism but not food intake,

and vice versa for the PVN [209]. Corroborating this, Burmeister et al. demonstrated that GLP-1 injection into the PVN but not the ARC reduced food intake, but injection of exendin-4 into either region potently suppressed appetite [210]. The latter study suggests that differences exist between the effects of native GLP-1 *versus* pharmacological GLP-1RAs, with only the latter achieving sufficient activation of ARC GLP-1Rs for hypophagia. Further supporting a role for the ARC in the anorectic effects of pharmacological agents, intra-ARC injection of liraglutide suppressed food intake and induced weight loss in rats [211], and the anorectic effects of peripherally administered liraglutide were abolished by ablation of, or intranuclear injection of exendin(9-39) into the ARC but not the PVN [179]. Taken together, these studies suggest that the ARC is a critical neural substrate for pharmacological GLP-1RAs, whereas the PVN is that for native GLP-1 (at least after intranuclear injection, though this may mimic the situation for endogenously-produced central GLP-1, and indeed the PVN is more densely innervated by PPG neurons than the ARC [204]). In addition to agonist differences, differences in the route of administration (intranuclear *versus* peripheral) may also affect access to, and activation of different nuclei. The ARC is located next to circumventricular organ the median eminence and is likely more accessible to blood-borne signals than the PVN that lies further dorsally. It may therefore be a more important substrate for circulating GLP-1RAs.

The abovementioned studies by Sandoval et al. and Burmeister et al. evidence the PVN to be sufficient for GLP-1R-induced hypophagia. Supporting a physiological role for the PVN, intranuclear injection of exendin(9-39) resulted in hyperphagia and weight gain, and dense, reciprocal innervation exists between the PVN and NTS [204], [212]. In addition to the ARC and PVN, the VMH, LHA and DMH have been implicated in GLP-1-mediated hypophagia. GLP-1 injection into all of these regions resulted in short latency, transient (1-2 hour) reductions in food intake [213], although only intra-LHA injections of liraglutide resulted in hypophagia and body weight loss at 24 hours in rats [211].

In summary, although it is likely that each of the mentioned hypothalamic nuclei play a role in GLP-1R-mediated appetite regulation, their relative importance in physiological and pharmacological settings remains to be precisely determined. Notably, recent studies involving nuclei-specific knockdown of the GLP-1R show that the arcuate, paraventricular and ventromedial hypothalamic nuclei are sufficient but not necessary for the acute anorectic effects of exendin-4 and liraglutide [210], [214]. These studies support a model wherein the anorectic effects of GLP-1R agonists involve the coordinated activity of multiple hypothalamic nuclei, rather than being reliant upon a single anatomical site.

1.6.2.2.3 Other midbrain and forebrain substrates

In addition to the classically studied hindbrain and hypothalamic regions, there is growing recognition of the importance of mesolimbic and hippocampal regions in GLP-1R-mediated hypophagia. The GLP-1R is expressed in the two key mesolimbic nuclei - the ventral tegmental area (VTA) and the nucleus accumbens core (NAc) [91] - and both regions receive innervation from PPG NTS neurons [215]. Supporting a physiological role for these regions, intranuclear injection of exendin(9-39) led to increased intake of chow, liquid sucrose meal, high-fat diet, or alcohol [215]–[218]. Correspondingly, intake of chow or high-fat diet was reduced by intra-VTA or intra-NAc injections of exendin-4, without eliciting rodent correlates of nausea (pica or conditioned taste avoidance) [215], [217], [219]. Reward-motivated responding for palatable food, alcohol or cocaine is also reduced by GLP-1R activation in

these regions [218]–[220]. Thus, mesolimbic circuitry appears to play a role in GLP-1R-mediated hypophagia by reducing the rewarding properties of food.

The hippocampus (HP) - a structure typically associated with learning and memory - is now acknowledged to play a role in the higher-order control of feeding behaviour [193]. The GLP-1R is expressed at high levels throughout the hippocampus [91], although the ventral region of the hippocampus (vHP) appears to be the key substrate for hippocampus-controlled food intake and appetitive behaviour. Accordingly, infusion of exendin-4 into the vHP reduced food intake and reward-motivated responding for palatable food, whilst exendin(9-39) had the opposite effects [221]. More recently, the lateral septum (LS) and dorsal lateral tegmental area have been implicated in GLP-1-mediated appetite regulation. Both regions are innervated by PPG NTS neurons, and infusion of exendin(9-39) into either of these regions increased food intake, whereas exendin-4 infusion led to hypophagia without inducing pica or conditioned flavour avoidance [222], [223]. Consistent with its association with reward and stress-related behaviours [224]–[226], GLP-1R activation in the LS plays a role in stress-induced hypophagia and the motivational aspects of feeding [222], [227]. Its activity appears to be coordinated with other GLP-1R-expressing regions, as an excitatory vHP to LS pathway, and an inhibitory LS to lateral hypothalamus pathway have been identified, both of which serve to suppress appetite [228], [229].

Thus the neurocircuitry involved in GLP-1R-mediated appetite regulation is emerging as a highly complex and widely distributed network, influenced by hedonic, stress, nausea, mnemonic and emotional cues in addition to homeostatic signals.

1.7 GLP-1-based treatments for T2DM and obesity

The multiple metabolic actions of GLP-1 pose GLP-1R agonism as an attractive approach for the treatment of T2DM and obesity. GLP-1's anti-diabetic actions include its induction of insulin secretion, inhibition of glucagon secretion, and enhancement of β -cell function. Moreover, its promotion of weight loss (due to its anorectic and pro-satiety effects) serves to increase insulin sensitivity, as well as combating obesity. An added benefit of GLP-1R agonism is the glucose-dependent nature of GLP-1's insulinotropism, thereby avoiding the risk of hypoglycaemia associated with the use of sulfonylureas or insulin analogues as T2DM treatments. However, the administration of native GLP-1 is therapeutically unviable given its short half-life due to rapid enzymatic degradation by DPP-4. The two major pharmacological methods to overcome this limitation are: (a) reducing the degradation of endogenous GLP-1 by inhibiting DPP-4; and (b) developing long-acting GLP-1R agonists with decreased susceptibility to elimination.

1.7.1 DPP-4 inhibition

The anti-hyperglycaemic effects of DPP-4 inhibition occur indirectly by increasing levels of endogenous, active GLP-1 in the circulation [230] and likely also locally in the gut and pancreas [231], [232]. The first DPP-4 approved for clinical use was sitagliptin, and many other agents including saxagliptin, vildagliptin and linagliptin have since been approved. DPP-4 inhibitors are oral, once- or twice-daily agents that cause HbA1c reductions of 0.6 to 1.1%, feature a good side effect profile, and are weight-neutral [233]. These agents may be prescribed as a monotherapy, but are mainly given in combination with metformin. Whilst incretin hormone GIP is also a substrate of DPP-4, the actions of

DPP-4 inhibitors are believed to be exerted primarily through augmentation of active GLP-1 levels [234]. Though widely prescribed, the modest 2- to 4-fold increase in circulating levels of GLP-1 achieved by this class of drugs sets an upper limit on their clinical efficacy [232].

1.7.2 Peptide GLP-1R agonists

In contrast to DPP-4 inhibitors, degradation- and clearance-resistant GLP-1RAs cause direct and supraphysiological stimulation of the GLP-1R to induce GLP-1-like anti-diabetic and anti-obesity effects. A number of approaches have been deployed to prolong the half-life of GLP-1RAs, namely sequence modification, the application of controlled release technologies, and conjugation to various moieties including fatty acids, albumin, and antibody fragment crystallisable (Fc).

1.7.2.1 Sequence modification & exendin-4-based analogues

Knowledge of the cleavage sites of DPP-4 and NEP-24.11 (see Section 1.5.1) in the GLP-1 sequence has facilitated the rational substitution of amino acids to prevent degradation. As DPP-4-mediated cleavage at Ala8-Glu9 is the main reason for native GLP-1's short half-life, a number of GLP-1RAs discussed below feature replacement of Ala8 [235]. Substitution of Ala8 with non-proteinogenic amino acid α -aminoisobutyric acid (Aib) as in semaglutide [236] and taspoglutide [237] leads to significant half-life extension. Likewise, albiglutide and dulaglutide feature an Ala8 to Gly8 substitution to minimise DPP-4 degradation [235].

In what could be viewed as a more extreme form of sequence modification, exendin-4 – a naturally-occurring peptide derived from the venom of the Gila monster *Heloderma suspectum* – shares 53% sequence homology with human GLP-1, is a potent GLP-1RA, and is resistant to DPP-4 by virtue of substitution of glycine for Ala2 and to NEP-24.11 via various other sequence changes throughout the molecule [235]. Its enzymatic resistance results in glomerular filtration in the kidneys being the main route of elimination [238], [239], and endows it with a half-life of approximately 26 minutes in humans after intravenous bolus delivery [240], and 2.4 hours after subcutaneous administration [241]. Exenatide, a synthetic form of exendin-4, was the first GLP-1RA clinically approved as a monotherapy for T2DM. Exenatide was first released under the brand name Byetta in 2009 as a twice-daily, subcutaneously-delivered formulation (referred to as “exenatide BID”) [235], and has been shown to cause HbA1c reductions of 0.9% and weight loss of 3.1 kg [242]. A once-weekly formulation – exenatide QW (brand name Bydureon) – that utilises a sustained-release drug delivery system was subsequently formulated and clinically approved in 2012. In the system, exenatide is encased in a poly(lactic-co-glycolic acid) polymer that slowly degrades to gradually release peptide. A head-to-head trial of exenatide BID and QW revealed the latter to cause superior glycaemic improvements (HbA1c reductions of 0.9% vs. 1.6%) and weight loss (-1.4 kg vs. -2.3 kg) [243]. Moreover, the incidence of nausea as an adverse event was reduced in the latter, likely due to reduced fluctuations in plasma drug concentration [243].

Lixisenatide (Adlyxin in US and Lyxumia in Europe) is another commercially successful exendin-4 analogue that features the substitution of the C-terminus proline with six lysine residues. Lixisenatide is prescribed as a once-daily formulation, and achieves similar glycaemic improvements to exenatide BID [244], [245].

1.7.2.2 Acylation

Acylation, the covalent attachment of a fatty acid, is the adopted approach for the half-life extension of liraglutide (Victoza) and semaglutide (Ozempic). Acylation protracts peptide half-life by both promoting peptide self-aggregation as well as causing binding to plasma albumin. The former results in the formation of an injection site depot, slowing uptake into the circulation [246], [247], whilst the latter reduces renal clearance and sterically hinders against enzymatic degradation [248], [249]. As a result, liraglutide and semaglutide boast half-lives of 13 hours and 7 days in humans, respectively, facilitating liraglutide's once-daily, and semaglutide's once-weekly administration [235]. Semaglutide's superior half-life relative to liraglutide is conferred by both the optimisation of the fatty acid and mechanism of conjugation, as well as the aforementioned Ala8 to Aib8 amino acid substitution [236]. Independent and head-to-head trials reveal both liraglutide and semaglutide to provide better glycaemic improvements than shorter-acting GLP-1RAs, with HbA1c reductions of 1.14% for liraglutide and 1.55% for semaglutide [250]–[252].

Whilst weight loss for liraglutide is comparable to shorter-acting GLP-1RAs at doses indicated for the treatment of T2DM, it is additionally licensed at a higher dose as an anti-obesity medication in non-diabetic patients (Saxenda) [253]. Comparison of liraglutide with other existing anti-obesity therapeutics (orlistat, lorcaserin, phentermine/topiramate, and naltrexone/bupropion) suggests similar weight loss efficacy of 5-10% [254]. Thus, as with other anti-obesity pharmacotherapies, liraglutide's weight-lowering effects are far from rivalling that of gold-standard bariatric surgery as a treatment for obesity. Compared to most other anti-obesity and T2DM medications, however, it offers the added benefit of cardiovascular improvements [255], [256]. Semaglutide also substantially lowers the risks of cardiovascular disease in T2DM patients [257] and, moreover, a recent phase II dose-finding trial evidenced semaglutide's enhanced weight loss efficacy relative to liraglutide [258]. Finally, semaglutide is being developed as an oral formulation through the use of an absorption enhancer N-[8-(2-hydroxybenzoyl)amino] caprylate to increase transcellular uptake in the stomach [259]. A recent phase II trial revealed low bioavailability following oral administration (~1%), though anti-hyperglycaemic and weight-lowering efficacy were matched to subcutaneously administered semaglutide, albeit at markedly different doses [260]. Phase III trials in T2DM patients testing oral semaglutide as a monotherapy [261] or in combination with other medications [262] have been completed and suggest clinically meaningful reductions in HbA1c, though further assessment is required.

1.7.2.3 Albumin and antibody fragment fusion

Albiglutide (Tanzeum) utilises an alternative approach to exploit the long circulating half-life and broad biodistribution of plasma albumin. The compound constitutes two copies of a GLP-1 analogue directly fused to human albumin, resulting in an *in vivo* half-life of 6-8 days [263]. To a similar effect, dulaglutide (Trulicity) consists of two monomers of a GLP-1 analogue fused to the human IgG4 fragment crystallisable (Fc) region, and has a half-life of 4.7 days [264]. Both GLP-1RAs are approved for once-weekly administration, and achieve comparable reductions in HbA1c to one another, and to liraglutide [265]–[268].

Albumin and Fc fragment conjugation extend peptide half-life through similar mechanisms. The increased molecular weight of the peptide-moiety compound decreases renal elimination, steric

hindrance from the bulky moiety reduces peptide proteolysis, and the engagement of a unique recycling pathway prevents lysosomal degradation in endothelial cells [235]. Regarding the latter mechanism, after endocytosis by endothelial cells, both albumin and the Fc fragment bind to the neonatal Fc receptor (FcRn) within acidic lysosomes and are recycled back to the plasma membrane where the neutral pH facilitates dissociation from the FcRn [269], [270]. Thus, they avoid endothelial cell-mediated lysosomal degradation.

Notably, albiglutide achieves poorer weight loss outcomes in head-to-head trials with other GLP-1RAs [268]. The increased molecular weight of albiglutide likely impedes transport across the blood brain barrier and prevents direct action upon GLP-1R-expressing anorectic neurons that is achieved by other GLP-1RAs [179].

1.8 Side effects of GLP-1-based treatments

GLP-1R-based treatments are most frequently associated with gastrointestinal side effects, including nausea, diarrhoea and vomiting. Of these, nausea is the most commonly reported adverse effect and its incidence directly correlates with GLP-1RA plasma levels [271]. As such, reports of nausea are less frequent for longer-acting GLP-1RAs given reduced fluctuations in peak drug plasma concentration, as compared to frequently-administered, shorter-acting GLP-1RAs. For example, clinical studies report the incidence of nausea to be between 33% and 57% for patients with twice-daily exenatide [272], 25% and 29% for once-daily liraglutide [252], [268], and between 9.1% and 9.9% for once-weekly albiglutide [266], [268]. Similarly, a head-to-head trial reported 35% of exenatide BID-treated, and 14% of exenatide QW-treated patients to experience nausea [243]. In addition to achieving more stable plasma drug concentrations, reduced nausea associated with longer-acting agents may also be related to reduced penetrance to CNS-located appetite regulating centres (specifically for albiglutide) [179], and a lack of effect upon gastric motility due to tachyphylaxis [273].

Concerns have been raised over an increased risk of pancreatitis with long-term GLP-1RA treatment. These arose from single patient case study reports [274], [275], though subsequent rodent studies examining a causal link have produced inconsistent results [276]–[280]. Likewise, inconsistency exists in human data. Whilst some post-marketing studies have reported an increased incidence of pancreatitis in patients receiving GLP-1-based treatments [281]–[283], meta-analyses of incretin trials have failed to identify an association [284], [285], and systematic reviews of preclinical and clinical data from both the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) also concluded that evidence for a causal association was insufficient [286].

Additional safety concerns associated with GLP-1RAs are injection site reactions, the development of anti-drug antibodies (reduced for GLP-1- *versus* exendin-4-based analogues due to increased sequence homology), and thyroid cancer. Regarding the latter, rodent studies established a mechanistic link between liraglutide-mediated GLP-1R stimulation in thyroid C-cells, calcitonin secretion and thyroid C-cell hyperplasia [287]. Whilst no correlation has been shown to exist between long-term liraglutide treatment, calcitonin secretion and incidence of thyroid cancer in both humans and cynomolgus monkeys [287], [288], liraglutide is still contraindicated in patients with a history of medullary thyroid cancer [235]. Species differences in the level of thyroid C-cell GLP-1R expression may account for these observations [287].

1.9 GLP-1R Signalling

The GLP-1R was first cloned in 1992 and identified as a member of the class B or secretin-like subfamily of G-protein-coupled receptors (GPCRs) [289]. GPCRs share a common architecture, consisting of an extracellular N-terminus (ECD), an intracellular C-terminus (ICD), and a transmembrane domain (TMD) of seven α -helices (TM1-7) linked by three extracellular (ECL1-3) and three intracellular loops (ICL1-3). Unique to class B GPCRs, however, is their possession of a large ECD that forms a three-layered α - β - β - α fold stabilised by inter-layer disulphide bonds [290]. This ECD is critical to the activation of class B GPCRs, as outlined in the widely accepted “two-domain” model of binding of class B GPCR ligands. In this model, the initial binding of the ligand C-terminus to the ECD facilitates subsequent binding of the ligand N-terminus to the TMD ligand-binding pocket. This secondary interaction causes receptor activation, inducing receptor conformational change and initiating intracellular signalling [290]. The initial receptor ECD-ligand C-terminus interaction has led to the ECD being referred to as an “affinity trap”. GLP-1R activation is believed to adhere to the two-domain model, though a recent study suggests that – and distinct from other class B GPCRs – the GLP-1R ECD is directly and persistently required for receptor activation, rather than acting as a transient affinity trap [291]. Following activation, the GLP-1R couples to multiple transducer and modulatory proteins, as outlined below, to result in a variety of intracellular consequences.

1.9.1 G-protein signalling by the GLP-1R

G-proteins, the canonical GPCR transducer proteins, are heterotrimeric proteins composed of $G\alpha$, $G\beta$ and $G\gamma$ subunits. Receptor activation leads to dissociation of the heterotrimer, with $G\alpha$ capable of signalling independently, whilst $G\beta$ and $G\gamma$ subunits remain as a heterodimer to exert their regulatory and signalling functions [292]. Whilst there is a growing appreciation for $G\beta\gamma$ -mediated functions [293], $G\alpha$ subunit-mediated signalling has dominated GPCR research. Four major classes of $G\alpha$ subunit exist (G_s , G_q , $G_{i/o}$ and $G_{12/13}$), differentiated by the effectors that they regulate (e.g. adenylyl cyclases, phospholipase C, mitogen-activated protein kinases) and the second messengers that they cause to accumulate (e.g. cyclic adenosine monophosphate, Ca^{2+} , inositol 1,4,5-triphosphate).

1.9.1.1 *Pancreas*

Studies in recombinant cell lines have demonstrated GLP-1R coupling to G_s , G_q , and $G_{i/o}$ forms of $G\alpha$ [294]–[296]. Of these, G_s signalling in pancreatic β -cells is the physiologically relevant pathway for insulin secretion. G_s -mediated activation of adenylyl cyclase results in accumulation of cyclic adenosine monophosphate (cAMP), which, in turn, directly activates protein kinase A (PKA) and cAMP-regulated guanine nucleotide exchange factor 2 (Epac2) [297], [298]. These two effectors coordinate insulin secretion by multiple processes, including ATP-sensitive K^+ channel closure [299], [300], opening of voltage-gated calcium channels [301], inhibiting membrane repolarisation by Kv channels [301], and eliciting Ca^{2+} -induced Ca^{2+} release from intracellular stores [302], [303]. The cumulative effect of these actions is increased cytosolic Ca^{2+} concentration, triggering exocytosis of insulin secretory granules. A physiological role for GLP-1R $G_{i/o}$ coupling in pancreatic β -cells is yet to be demonstrated, though a role for G_q coupling in insulin secretion has been suggested [304].

1.9.1.2 Neurons

GLP-1R signalling in both peripherally- and centrally-located neurons plays a crucial role in appetite-suppressive effects. Despite this, and in contrast to pancreatic β -cells, GLP-1R intracellular signalling in neurons has not been extensively characterised. This likely reflects the complexity of the neurocircuits involved in GLP-1-mediated hypophagia. GLP-1R-expressing neuronal subpopulations are widely distributed and phenotypically distinct in their morphology, connectivity, and expression of receptors and neuropeptides. The critical GLP-1R-associated signalling pathways engaged in one brain region, and subsequent pre- or post-synaptic neuronal changes that occur, may therefore be different from those elsewhere [305]. Despite these challenges, some insight into hypophagia-related signalling downstream of the GLP-1R has been garnered for specific brain regions.

The anorectic effects of GLP-1R agonism in the NTS are mediated by cAMP/PKA signalling. Appetite suppression following administration of exendin-4 into the 4th ventricle (i.e. intracerebroventricularly) was dependent upon PKA-mediated suppression of adenosine monophosphate protein kinase (AMPK) activity and concomitant activation of p44/42 mitogen-activated protein kinase (p44/42 MAPK, otherwise known as ERK1/2) [306]. These PKA-dependent effects were purported to promote Ca^{2+} -dependent depolarisation of the GLP-1R expressing neurons [306]. Of note, a temporal discrepancy existed between PKA signalling in the NTS and the associated behavioural effects. Whilst PKA activity peaked at 10 minutes post exendin-4 administration and returned to baseline by 30 minutes, food intake suppression was observed at 3 hours post administration [306]. These results suggests that longer term, downstream signalling changes are required for behavioural effects (e.g. cAMP response element-binding protein (CREB)-mediated transcriptional effects), as well as the recruitment of additional CNS appetite-regulating nuclei.

PKA-dependent signalling is also critical to GLP-1R-induced plasticity in NTS-to-PVN anorectic neurocircuits [307]. GLP-1R activation in PVN corticotropin-releasing hormone (CRH) neurons in *ex vivo* brain slices leads to PKA-dependent phosphorylation of serine S845 on GluA1-containing AMPA receptors (AMPA), their subsequent insertion into the postsynaptic membrane, and an increase in excitatory synaptic strength. These changes appear physiologically relevant, as the anorectic effects of PVN-administered exendin-4 were abolished in mice in which PKA inhibitors were injected into the PVN, the trafficking of GluA1 AMPARs in the PVN was blocked, or in mice expressing a serine S845 phospho-deficient form of the GluA1 subunit [307]. The observed increases in synaptic strength were not dependent upon Epac activity, however.

GLP-1R-mediated changes in synaptic signalling have also been demonstrated to occur in other brain regions associated with GLP-1's anorectic effects, namely the VTA [308], [309], NAc [310], HP [311], [312], LHA [313], and ARC [179]. However, the associated intracellular signalling pathways were not examined in these studies. Moreover, the dependence of GLP-1's anorectic effects upon the identified neuronal signalling changes has only been demonstrated *in vivo* for the VTA and NAc [309], [310]. Thus, whilst progress has been made in understanding GLP-1R intracellular signalling in neurons, and G_s (i.e. cAMP/PKA) signalling appears to play a critical role [306], [307], knowledge in the area is somewhat underdeveloped. In particular, the physiological relevance of many changes observed in *in vitro* and *ex vivo* studies remains to be established.

1.9.2 The role of β -arrestins in GLP-1R signalling and trafficking

The β -arrestins are multifunctional modulatory proteins that were first described for, and still best known for their role in GPCR signal termination [314]. After GPCR activation, phosphorylation of the receptor intracellular domain by G-protein receptor kinases (GRKs) results in the recruitment of β -arrestins to the receptor. β -arrestins induce GPCR desensitisation (i.e. reduced receptor responsivity to subsequent stimulation) by sterically hindering receptor engagement with G proteins [315], and also cause GPCR internalisation by acting as a scaffold for the assembly of clathrin-coated endocytic vesicles [316], [317]. In addition to these canonical roles, β -arrestins can initiate intracellular signalling by scaffolding components of the ERK1/2 cascade [318]. It should be noted that there has been recent controversy in the field, with studies challenging [319], [320] and subsequently defending [318] the existence of bonafide β -arrestin-mediated signalling. These findings highlight the difficulty of studying proteins that serve diametrically opposed functions in ERK1/2 signalling (i.e. inhibiting G-protein-mediated ERK1/2 signalling by promoting GPCR desensitisation, whilst serving as a scaffold to initiate ERK1/2 signalling), and emphasise the importance of the model system (e.g. siRNA-mediated knockdown *versus* CRISPR-edited cells) used for the study of these proteins [318].

Two isoforms of β -arrestin exist - β -arrestin-1 and β -arrestin-2 – that share 78% sequence homology and mediate both distinct and common cellular functions. The GLP-1R associates with both isoforms, and a role for β -arrestins in both GLP-1R signal generation and signal termination has been demonstrated [321]–[323]. A role for β -arrestin-1 in both insulin secretion and anti-apoptotic effects in pancreatic β -cells has been reported. Regarding the former, knockdown of β -arrestin-1 in pancreatic β -cells *in vitro* caused a reduction of GLP-1-stimulated cAMP production, ERK1/2 phosphorylation and insulin secretion [322]. Regarding the latter, ERK1/2 signalling via β -arrestin-1 activates ribosomal s6 kinase (p90RSK), which in turn phosphorylates and inactivates pro-apoptotic protein Bcl-2-associated death promoter (BAD) [324].

After ligand binding, the GLP-1R undergoes rapid homologous and heterologous desensitisation [325], [326], and also internalises with fast kinetics as a ligand-bound complex [327]. A role for β -arrestins in GLP-1R signal termination has been suggested upon the basis that GLP-1R agonists with a reduced propensity for β -arrestin recruitment (“biased” agonists, as discussed below) exhibit prolonged cAMP signalling and insulin secretion in pancreatic β -cells [323]. This was associated with reduced agonist-induced GLP-1R internalisation and increased recycling. The relative roles for β -arrestins in GLP-1R desensitisation and internalisation were probed in this study by conducting dual siRNA-mediated knockdown in pancreatic β -cell lines. Exendin-4-induced insulin secretion was consistently enhanced after dual knockdown, although only a modest decrease in GLP-1R internalisation was observed. Similar findings of prolonged signalling (cAMP accumulation) but a partial effect upon internalisation were observed in β -arrestin-less CRISPR/Cas9-modified HEK293 cells, excluding the possibility of incomplete siRNA-mediated knockdown in β -cell lines. Thus, whilst these findings suggest a role for β -arrestins in both GLP-1R desensitisation and internalisation, they point to a more important role for these proteins in the former function.

The subtle effects of dual β -arrestin knockdown on GLP-1R internalisation partly aligns with previous observations of siRNA- [322] or dominant negative construct-mediated inactivation of only β -arrestin-1 [328] having no effect upon GLP-1R internalisation, and simultaneously suggest a greater role for β -arrestin-2 in this function, as has been previously indicated [321]. The fact that the GLP-1R

undergoes rapid and extensive internalisation despite modest β -arrestin contributions to this event is perhaps reconciled by the GLP-1R's engagement with β -arrestin- and clathrin-independent, caveolin-mediated forms of endocytosis [328], [329].

It should be noted that the inverse relationship between β -arrestin recruitment and insulin secretion observed in Jones et al. is initially at odds with Sonoda et al.'s findings of β -arrestin-1-mediated insulin secretion [322], [323]. This apparent inconsistency can be explained by differences in the utilised duration of GLP-1R stimulation. Jones et al. used prolonged agonist incubations to mimic *in vivo* drug exposure, whilst Sonoda et al. used brief stimulation, more closely mirroring physiological GLP-1 effects. Thus, although a positive role for β -arrestin-1 in insulin secretion may occur with acute GLP-1R stimulation, a negative role for either, or both β -arrestins (due to signal termination) supersedes this effect with prolonged agonist exposure.

Finally, the dogma that GPCR internalisation is associated with signal termination has been challenged. There are now numerous reports for many GPCRs of sustained signalling from endocytic compartments [330]–[332], including the GLP-1R [333]. Moreover, β -arrestin-mediated GLP-1R internalisation and subsequent recycling to the plasma membrane appears to be an important mechanism for receptor resensitisation [323]. Consistent with both of these mechanisms of sustained signalling, chemical [333] or genetic [334] inhibition of dynamin – a GTPase involved in both clathrin- and caveolin-mediated endocytosis – results in blunted GLP-1R signalling.

1.10 Biased agonism

Traditional pharmacology subscribed to a “two-state” model of receptor activation, wherein ligand binding to an inactive receptor could cause it to assume an “active” structural conformation and elicit a cellular response [335]. Ligands were classified as full agonists, partial agonists, inverse agonists, or antagonists based upon their propensity to stabilise the active or inactive receptor form. Though it has long been recognised that most GPCRs couple to multiple transducer and modulatory proteins (G-proteins, β -arrestins, GRKs), the binary nature of the two-state model dictated that ligand binding to a GPCR led to equal activation of all coupled pathways (all “on” or “off”). More recently, it has become appreciated that ligands are capable of selectively activating different intracellular pathways by stabilising different active receptor conformations. This phenomenon has been termed “biased agonism” or “functional selectivity” [336], [337]. Contrary to the two-state model, structural studies using nuclear magnetic resonance (NMR) spectroscopy have confirmed the existence of multiple active receptor conformations, each recruiting different intracellular transducers with differential efficiency [338], [339].

An agonist that displays this property is known as a “biased agonist”. The identification of biased agonists involves the comparison of signalling responses in at least two pathways, expressed relative to those of a reference agonist. It is important to note that measured agonist bias reflects both true “ligand bias” (the biomolecular ligand-receptor interaction that stabilises a specific receptor conformation, a test system-independent property) as well as cell- and assay-specific properties that influence the observed response (referred to as “system bias”¹ and “observational bias”, respectively).

¹ The term “system bias” has been used in the literature to refer solely to cell-specific effects [340], [342], as well as collectively to cell-specific and other assay-specific effects [341]. Here the former definition will be used.

The latter two phenomena mean that bias is only meaningful when described relative to a reference agonist tested in parallel [336]. For reliable identification, different agonists should be directly compared (i) in the same cell line to avoid differences in the level of receptor or transducer expression, or receptor-transducer coupling (“system bias”); (ii) using matched assays to avoid differences in signal amplification or assay sensitivity (“observational bias”); (iii) using identical assay conditions (e.g. assay medium) to minimise non-receptor influences on cellular responses; and (iv) using matching assay duration to accurately compare signal accumulation [337], [340]. Additionally, the existence of downstream crosstalk between intracellular pathways means that assays of receptor-proximal signals should be used to successfully isolate individual pathway responses [337]. Thus, accurately quantifying ligand bias is a non-trivial exercise. However, multiple recent reviews implore that it is conducted properly in order to increase the translation of biased agonists identified *in vitro* to successful *in vivo* therapeutics [336], [340]–[342].

1.10.1 Therapeutic applications of biased agonism

As alluded to above, the application of biased agonism has therapeutic utility. GPCR activation leads to multiple cellular responses, some of which are linked to desired drug effects and others to unwanted side effects. As such, biased agonism provides the possibility of selectively modulating these responses to enhance drug efficacy and minimise adverse effects – that is, to produce optimised therapeutics.

A prototypical example of exploiting signal bias to improve therapeutics involves μ -opioid receptor (MOR) agonists. MOR agonists (e.g. morphine, fentanyl) provide powerful analgesia but are associated with side effects including respiratory depression, nausea, vomiting, and sedation. The role of β -arrestin 2 in both mediating these side effects and limiting analgesic efficacy (the latter by promoting MOR desensitisation and internalisation) has been robustly demonstrated [337]. MOR agonist administration to β -arrestin 2 knockout mice [343], [344] or rodents with RNAi-induced β -arrestin 2 knockdown [345], [346] results in enhanced analgesia, reduced respiratory depression, and reduced constipation. These findings instigated large-scale screens for MOR agonists biased away from β -arrestin recruitment and towards G-protein signalling. From these, G-protein-biased oliceridine (formerly TRV130) was identified: a small molecule agonist with matched potency and efficacy to morphine for G-protein signalling, but markedly reduced β -arrestin recruitment. Preclinical studies evidenced its improved side effect profile compared to morphine at equianalgesic doses [347], and these findings have now been replicated in clinical phase IIB [348] and phase III trials [349]. Whether oliceridine’s improved profile is sustained with chronic treatment in humans has not yet been evidenced. However, its development represents a powerful example of the application of biased agonism to refine existing therapeutics. Of note, the search for further improved MOR agonists has continued, with an *in silico* screen of over 3 million molecules for MOR docking identifying PZM21, another strongly G-protein-biased agonist with an improved side effect profile *in vivo* [350].

This approach of identifying intracellular pathways associated with desirable or undesirable drug effects and subsequently designing “optimised” biased agonists has been applied to several other disease-relevant receptor systems. These include: dopamine D2 receptors for schizophrenia [351], nicotinic acid receptors for hyperlipidaemia [352], parathyroid hormone receptors for osteoporosis [353], serotonin receptors for depression, addiction and schizophrenia [354]–[356], and adenosine receptors for cancer, inflammation and cardiovascular disorders [357]. Additionally, some clinically

established therapeutics have been retrospectively shown to act as biased agonists. For example, carvedilol is a β -blocker used to treat cardiac failure that, in addition to exhibiting the standard β -blocker antagonism of G-protein signalling at β 1- and β 2-adrenergic receptors, also shows β -arrestin-dependent signalling that further promotes cardiomyocyte survival and may underlie its enhanced clinical efficacy relative to other members of the drug class [358]–[360].

However, the application of biased agonism is not always therapeutically successful, as captured by the development of biased ligands of the angiotensin II type 1 receptor (AT1R). Biased ligand TRV027 exhibited standard anti-hypertensive effects via antagonism of AT1R G-protein signalling, but – unlike in-clinic unbiased AT1R agonists – also improved cardiac contractility via β -arrestin signalling. Despite showing promising and consistent results in rodent [361] and canine models [362], a clinical phase II study failed to meet its primary endpoint [363]. Its failure may be due to the recently coined concept of “dynamic bias”: where pathologic, iatrogenic or even different physiologic states may alter the signalling landscape, including membrane composition, and the stoichiometric ratios of receptors, transducer proteins and modulatory proteins [336], [342]. Congestive heart failure, for which TRV027 missed its primary endpoint, is characterised by changes in G-protein, β -arrestin and GRK expression [364]; these disease-induced signalosome changes may have negated the clinical relevance of AT1R biased signalling [342]. Given the impact of dynamic bias, the application of biased agonism to drug development is expected to be most successful for diseases for which the pathological mechanisms are robustly understood [336], [342].

1.10.2 Biased signalling at the GLP-1R

The existence of biased agonism at the GLP-1R has been robustly established. The first demonstration revealed pharmacological GLP-1RA exendin-4 and endogenous ligands GLP-1(7-36)NH₂ and GLP-1(7-37) to preferentially induce cAMP accumulation over Ca²⁺ mobilisation and ERK1/2 phosphorylation [365]. By contrast, oxyntomodulin (OXM) was biased towards ERK1/2 phosphorylation, suggesting that different endogenous ligands may engender different physiological responses after GLP-1R activation [365]. Later studies assessing additional signalling pathways have demonstrated OXM and exendin-4 to be biased towards β -arrestin recruitment relative to GLP-1 (the latter more modestly than the former), as well as confirming the bias of OXM towards ERK1/2 phosphorylation [366], [367]. Interestingly, OXM's increased induction of ERK1/2 phosphorylation relative to GLP-1 appears to be related to its ability to cause unique, cytosolically-located ERK1/2 activity, in addition to the standard nucleus-located ERK1/2 activity produced by GLP-1 and exendin-4 [367].

Early studies also demonstrated the co-binding of small molecule allosteric modulators to alter the pathway engagement of GLP-1R orthosteric agonists in ligand-dependent manner [365], [368]. For example, “compound 2” augmented G_s signalling for OXM, but not for exendin-4, GLP-1(7-36)NH₂ or GLP-1(7-37), whilst enhancing β -arrestin responses for all agonists [365], [368]. By contrast, quercetin selectively modulated Ca²⁺ responses to exendin-4 and truncated forms of GLP-1, but not to OXM [365]. Thus, biased signalling at the GLP-1R can be both directly induced by orthosteric ligands, as well as modulated by allosteric small molecule ligands.

Moreover, signal bias at the GLP-1R can be produced by alterations to the sequence of orthosteric peptide agonists. For example, changes to the GLP-1 peptide backbone by substituting α - with β -

amino acid residues altered agonist bias between G-protein signalling and β -arrestin recruitment [369]. Similarly, an N-terminally modified form of exendin-4 – “P5” – exhibited bias towards G-protein signalling relative to exendin-4, causing comparable cAMP accumulation but markedly reduced β -arrestin recruitment [370]. This *in vitro* signal bias translated to favourable effects *in vivo*, with P5 displaying enhanced anti-hyperglycaemic effects in intraperitoneal glucose tolerance tests (IPGTTs) relative to exendin-4 [370]. In characterising the physiological consequences of P5’s signal bias, Zhang et al. provided the first demonstration of the potential therapeutic utility of signal bias at the GLP-1R to enhance anti-diabetic medications.

P5 featured multiple amino acid changes to the exendin-4 sequence, with amino acids 1-8 being replaced by a novel sequence of 7 amino acids [370]. More recently, it has been demonstrated that single amino acid changes to the exendin-4 sequence can confer signal bias at the GLP-1R [323]. Characterisation of signal bias (G-protein signalling *versus* β -arrestin recruitment), agonist-induced GLP-1R internalisation, and insulin secretion in pancreatic β -cell lines *in vitro* was conducted for a panel of exendin-4 analogues with single amino acid substitutions proximal to the N-terminus. Consistent with a role for β -arrestins in receptor desensitisation and internalisation, G-protein-biased agonists (i.e. with reduced β -arrestin recruitment) consistently caused reduced GLP-1R internalisation, prolonged G-protein signalling, and enhanced maximal insulin secretion *in vitro* after extended stimulation, whereas β -arrestin-biased agonists resulted in the opposite effects [323]. These findings suggest that slowly-internalising, G-protein-biased agonists maintain a larger fraction of responsive receptors at the cell surface, facilitating prolonged signalling and secretory responses. Investigation of the therapeutic potential of a favoured G-protein-biased agonist – “exendin-phe1”, featuring a phenylalanine substitution at position 1 – evidenced its enhanced anti-hyperglycaemic effects relative to exendin-4 in IPGTTs, specifically with a delay between agonist administration and glucose challenge to allow the trafficking- and signalling-related advantages of G-protein-biased exendin-phe1 to emerge [323]. Interestingly, despite exendin-phe1’s improved anti-hyperglycaemic effect, it produced comparable anorectic effects to exendin-4, suggesting a divergence between the manifestations of GLP-1R biased signalling between these two physiological systems.

Recent studies from the Sexton laboratory have provided valuable insights into the structural basis for biased agonism at the GLP-1R. In each, systematic mutagenesis of residues in different receptor regions was conducted, followed by testing for altered affinity, and characterisation of the pharmacological responses (cAMP accumulation, intracellular Ca^{2+} mobilisation, ERK1/2 phosphorylation) to GLP-1, exendin-4 and oxyntomodulin. These differentially biased ligands consistently showed sensitivity to different sets of mutations, supporting the existence of pathway-specific active GLP-1R conformations. In particular, these studies have revealed: ligand-specific patterns of hydrogen bonding between polar residues in the transmembrane domains of the GLP-1R [371]–[373]; ligand-specific modes of engagement at the GLP-1R extracellular face [366]; and ligand-specific roles for the far N terminus and linker region between transmembrane domain 1 and the GLP-1R extracellular domain in receptor signalling [374]. Overlaying the combined results of these mutagenic analyses onto recently resolved active and G_s protein-bound GLP-1R structures [375]–[377] has allowed the direct identification of pathway-specific receptor conformations [374]. Whilst cAMP signalling and Ca^{2+} mobilisation features conformational changes to transmembrane helices 5 and 6 and re-ordering of extracellular loop 2, ERK1/2 phosphorylation relies upon ordering and packing of residues in transmembrane helices 1 and 7 [374]. Thus, there is a rapidly growing body of

knowledge pertaining to the structural basis of biased agonism at the GLP-1R, the findings of which may facilitate the design of favourably-biased therapeutics.

1.11 Conclusion

GLP-1RAs are an attractive treatment modality for T2DM given their dual insulinotropic and anorectic effects. Moreover, the latter has led to their recent licensing as independent anti-obesity medications. Current evidence suggests that they have a good side effect profile, with the main complication being nausea at treatment initiation.

Biased agonism describes the ability of ligands, usually for GPCRs but also as applied to non-GPCR targets [378], to cause the selective activation of particular intracellular pathways, leading to distinct cellular and, in cases, physiological outcomes. Importantly, the phenomenon transcends *in vitro* contexts and may be leveraged to develop optimised therapeutics with increased efficacy and/or reduced side effects.

Recent demonstrations have evidenced the ability of biased GLP-1R agonists to cause enhanced anti-hyperglycaemic effects. Intriguingly, this comes without concomitant increases in anorectic efficacy, but with reduced signs of nausea. Thus, a divergence appears to exist between the physiological manifestations of biased GLP-1R signalling in the contexts of glucoregulation and appetite regulation. The primary focus of this thesis is an investigation into the physiological mechanisms underlying this apparent divergence.

Within the GLP-1RA drug class, acylation has emerged as a successful means of half-life extension, leading to prolonged effects and reduced frequency of dosing. Biased GLP-1RAs with improved anti-hyperglycaemic effects may therefore be further optimised by acylation. A secondary focus of this thesis is thus to assess the impact of acylation upon the *in vitro* and *in vivo* properties of biased GLP-1RAs.

2 Methods

2.1 Peptides

Exendin-4 and exendin(9-39) were synthesised by Bachem, Switzerland. Custom peptides, including peptide-lipid conjugates and some fluorescent peptide conjugates, were obtained from WuXi AppTec, China. Fluorescent peptide conjugates were also prepared by a collaborator (Dr Maria Shchepinova, Imperial College London, further details are provided below). Sequences of peptides are given in Appendix 1. Experiments were performed with peptide freshly prepared from lyophilised aliquots.

2.2 Synthesis of Fluorescently-Tagged Peptides by Click Chemistry

Biased GLP-1RAs were obtained bearing the non-proteinogenic, alkyne-amino acid L-propargylglycine ("pra") at position 12 (substituting endogenous lysine residue). Sulfo-cyanine7 azide (Lumiprobe, USA) or azide-functionalised Vivotag-S 750 Fluorochrome (Perkin Elmer, USA) were conjugated to the pra residue by copper-catalysed alkyne-azide cycloaddition (CuAAC) "click" chemistry by Dr Maria Shchepinova, Imperial College London. HPLC-purified conjugates were resuspended in 0.9% NaCl, with addition of 1-2 µl of 1M NaOH to aid peptide dissolution. Concentrations were confirmed by absorbance readings at 280 nm on a Nanodrop OneC (Thermo Scientific, USA) and also using the molar absorptivity constant for each fluorophore and absorbance measurement at the appropriate wavelength determined using a SpectraMax i3X plate reader (Molecular Devices, USA).

2.3 Cell Culture

PathHunter CHO-GLP-1R β -arrestin-2 reporter cell lines (DiscoverX, USA) were maintained in Ham's F-12 Nutrient Mixture (Gibco, USA) supplemented with 10% FBS, 1 mM L-glutamine, 1 mM sodium pyruvate, 1% penicillin/streptomycin, 1 mg/ml G418, and 0.4 mg/ml hygromycin B. INS-1 832/3 cells (a kind gift from Professor Christopher Newgard, Duke University) were maintained in RPMI-1640 (Gibco, USA) including 11 mM D-glucose and supplemented with 10% FBS, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 0.05 mM 2-mercaptoethanol and 1% penicillin/streptomycin. MIN6B1 cells were maintained in DMEM containing 25 mM D-glucose and supplemented with 15% FBS, 0.05 mM 2-mercaptoethanol and 1% penicillin/streptomycin. mHypoA POMC/GFP3 cells [379] (Cedarlane Laboratories, CA) were maintained in DMEM containing 25 mM D-glucose and supplemented with 10% FBS and 1% penicillin/streptomycin. Stably transfected HEK293-SNAP-GLP-1R cells were maintained in DMEM containing 25 mM D-glucose and supplemented with 10% FBS, 1% penicillin/streptomycin and 0.5 mg/ml G418. Stably transfected CHO-K1-SNAP-GLP-1R cells were maintained in DMEM containing 25 mM D-glucose and supplemented with 10% FBS, 1 mM sodium pyruvate, 20 mM HEPES, 2% non-essential amino acids, 1% penicillin/streptomycin and 0.5 mg/ml G418.

2.4 Animal Studies

Male C57Bl/6 mice aged 8-16 weeks and male Wistar rats aged 8-10 weeks (Charles River, Margate, Kent, UK) were maintained under the following conditions unless otherwise stated: 21-23°C, 12:12 hour light-dark cycle, ad libitum access to water and standard chow diet (RM1, Special Diet Services Ltd, Witham, Essex, UK) or 60 kcal% fat high-fat diet (D12492, Research Diets, USA). Rats were individually caged; mice were either group caged (4 per cage) for glucose tolerance tests, or

individually caged for food intake studies. All animals were regularly handled to mitigate non-specific stress effects.

2.5 Lateral Ventricle Cannulations

Lateral ventricle cannulations were performed with Dr David Ma, Imperial College London. Male C57Bl/6 mice aged 7.5 weeks were anaesthetised with 2 L/min O₂ and 4-5% isoflurane in an induction chamber until loss of the pedal reflex was confirmed. Animals were intraperitoneally administered prophylactic antibiotics amoxicillin (25 mg/kg) and flucloxacillin (25 mg/kg), and analgesics carprofen (4 mg/kg) and buprenorphine (0.12 mg/kg). Fur was shaved from the dorsal cranial area caudal to the eyes and lacrilube (Allergan, UK) was applied to the eyes to prevent keratitis. Mice were positioned on a stereotactic frame (Model 900, David Kopf Instruments, USA) using ear bars and incisor bar. A mouse anaesthetic mask (Model 907, David Kopf Instruments, USA) was fitted to deliver 2 L/min O₂ and 1.5-2.5% isoflurane; the level of anaesthesia was adjusted throughout the procedure to ensure appropriate depth of anaesthesia based upon respiratory rate and pedal reflex. The shaved skin was cleaned with 10% povidine-iodine solution and a 1.5 cm longitudinal incision was made on the dorsal cranial surface to expose the cranium. Coordinates of bregma were recorded and used to calculate rostrocaudal and mediolateral coordinates of the left lateral ventricle (0.4 mm caudal from bregma, 0.1 mm lateral from bregma). A unilateral hole of 0.5 mm diameter was drilled in the cranium using a stereotactically mounted electric drill (Foredom Electric Co., USA) for insertion of the cannula. Four additional indentations were drilled in the cranium in a square configuration around cannula hole, and stainless steel screws (Antrin Miniature Specialities, USA) partially inserted into the indentations. A stainless steel 26 gauge cannula projecting 2.2 mm ventrally (Plastics One, USA) was inserted into the drilled cannula hole using a stereotactically mounted clamp. Dental cement (Kemdent, UK) was applied around the cannula in the area enclosed by the four screws to secure the cannula in place. Once dry, the clamp was removed and a 33 gauge stainless steel dummy cannula (Plastics One, USA) was inserted into the cannula. Mice were administered 0.3 ml of warmed 0.9% NaCl solution and allowed to recover in a 32°C chamber. Once the righting reflex was regained, mice were returned to their cages with softened RM1 diet, and were monitored for the week post-surgery (daily body weight measurements and administration of 4 mg/kg carprofen). After the 7 day recovery period, animals received daily handling (including removal and reinsertion of the dummy cannula) and sham 0.9% NaCl intraperitoneal injections for 4 days.

2.6 Acute Food Intake Studies

2.6.1 Peripheral Administration Feeding Studies

Animals were randomised into weight-matched groups for all studies. For regular food intake studies, animals were fasted for 16 hours overnight. At the onset of the light phase, 100 µl of reconstituted peptide or vehicle (0.9% NaCl) was administered by intraperitoneal (mice) or subcutaneous (rats) injection at the indicated weight-adjusted dose and *ad libitum* access to diet was reinstated. Diet was weighed at the indicated time points.

For dose response experiments, mice were fasted 10 hours prior to the onset of the dark phase. Animals were administered 100 µl of peptide or vehicle (0.9% NaCl) by intraperitoneal injection either at the onset of the dark phase ("acute" testing) or 6 hours prior to the onset of the dark phase

(“delayed” testing) at the indicated weight-adjusted dose. Diet was returned at the onset of the dark phase and weighed one hour later.

2.6.2 Central Administration Feeding Studies

A four-way crossover design was used for central administration studies. The order of peptide administration was randomised to prevent a treatment sequence effect. Animals were fasted for 16 hours overnight. Peptide (135 pmol/kg) or vehicle (water for injection) was delivered to conscious mice by a 33 gauge stainless steel injector (Plastics One, USA) inserted into and projecting 0.5 mm ventral to the end of the indwelling lateral ventricle cannula. An electrical infusion pump (Model A55-1111, Harvard Apparatus, USA) was used to deliver 0.5 µl of solution at a flow rate of 12 µl/hr via a closed water system consisting of a 10 µl, 26 gauge Hamilton syringe (Model 801 RN SYR, Hamilton, USA) attached via a 0.58 mm internal diameter polythene tube (Plastics One, USA) to the injector. The injector was left *in situ* for two minutes post-administration to prevent backflow of solution, before the injector was removed and dummy cannula reinserted. Diet was returned post-injection and weighed at the indicated time points. A minimum of 72 hours washout was enacted between studies.

Control studies confirming that the effects of centrally administered exendin-4 are CNS-mediated were conducted similarly, but with intraperitoneal administration of exendin(9-39) (200 nmol/kg) or vehicle (0.9% NaCl) immediately after central administration of exendin-4 (135 pmol/kg) or vehicle (water for injection).

2.7 Intraperitoneal Glucose Tolerance Tests

Mice were randomised into weight-matched groups for all studies. Mice were fasted for 5-8 hours prior to testing. For acute testing (co-administration of peptide and glucose), peptide was resuspended in 20% dextrose (D-glucose) to the indicated weight-adjusted dose of peptide, and administered by intraperitoneal injection with simultaneous delivery of a weight-adjusted 2 g/kg dose of glucose. For delayed testing, animals were administered 100 µl of peptide (resuspended in 0.9% NaCl) or vehicle (0.9% NaCl) intraperitoneally at the indicated weight-adjusted dose at the indicated time prior to 2 g/kg glucose challenge (typically 6 hours prior for non-acylated peptides, and 72 hours for acylated peptides). Blood glucose measurements were taken with a GlucoRx Nexus glucometer (TaiDoc Technology, Taiwan) immediately before glucose challenge and at the indicated time points thereafter. For glycaemic dose-response experiments, both acute and 6 hour delayed GTTs were conducted on the same day (peptide administration for the acute GTT being that for 6 hour delayed GTT).

2.8 Pharmacokinetic studies

Ad libitum fed mice were administered peptide by intraperitoneal injection or via a lateral ventricle cannula at the indicated weight-adjusted dose. At the indicated time points, blood samples were collected by tail vein bleeds into lithium heparin-coated microvette tubes (Sarstedt, Germany). Plasma was separated by centrifugation for 8 minutes at 10 000 g, 4°C and stored at -80°C. Plasma peptide concentration was measured by an exendin-4 ELISA (Phoenix Pharmaceuticals, USA) according to the manufacturer’s instructions.

2.9 Cyclic AMP Assays

Cyclic AMP was measured in a number of cell types, with the protocol and stimulation conditions specific to each (see below). Cyclic AMP accumulation was determined using the homogenous time resolved fluorescence (HTRF)-based cAMP Gs Dynamic kit (Cisbio).

2.9.1 PathHunter CHO-GLP-1R β -arrestin-2-EA Cells

PathHunter CHO-GLP-1R β -arrestin-2 cells in culture were detached with 0.02% EDTA (Sigma) and plated at 10 000 cells per well in 10 μ l of serum-free F12 medium in opaque half-area 96-well plates (Grenier Bio-One). For assays using acylated peptides, no BSA was added to serum-free medium for peptide resuspension. Cells were stimulated with 10 μ l of peptide made up in serum-free F12 medium at the indicated concentration in duplicate. After a 30 minute incubation at 37°C, 5% CO₂ with plates uncovered, 10 μ l of cAMP-d2 working solution followed by 10 μ l of anti-cAMP cryptate working solution was added to all wells (HTRF detection reagents made up in Lysis buffer 2 according to the manufacturer's instructions). Plates were incubated at room temperature for 60 minutes before and HTRF was read on a SpectraMax i3x (Molecular Devices) with the following settings: 340 nm excitation, 616 nm and 665 nm emission, 500 μ s integration time, 30 μ s delay, and 6.28 mm read height. cAMP production was determined by interpolation of a standard curve (run according to the manufacturer's instructions), and data analysed by applying four-parameter logistic fit in GraphPad Prism 7.03.

2.9.2 mHypoA POMC/GFP3 Cells

2.9.2.1 Dose Response

mHypoA POMC/GFP3 cells were seeded into clear, 96-well plates at 5000 cells per well in complete medium and allowed to adhere overnight. The following day, medium was removed from wells and 40 μ l of peptide made up in serum-free DMEM with 0.1% BSA and 500 μ M 3-isobutyl-1-methylxanthine (IBMX) was added to wells at the indicated concentrations, in duplicate. After a 5 minute incubation at 37°C, 5% CO₂ with plates uncovered, 40 μ l of Lysis Buffer 2 was added to all wells. Plates were gently agitated on a plate shaker for 5 minutes, before 20 μ l of lysate from all wells was added to opaque, 96-well, half area plates. 10 μ l of cAMP-d2 working solution followed by 10 μ l of anti-cAMP cryptate working solution was added to all wells (HTRF detection reagents made up in Lysis Buffer 2 according to the manufacturer's instructions). Plates were incubated at room temperature for 60 minutes before HTRF signal was read and analysed as described above.

For experiments with antagonist exendin(9-39), 10 μ M exendin(9-39) or no antagonist was included in the cell stimulation medium so that agonist and antagonist would be applied together, and the experiment performed as above.

2.9.2.2 Prolonged Incubation Dose Response

Prolonged incubation assays were performed similarly to acute dose response assays, but with cells stimulated in 100 μ l of peptide made up in complete DMEM with 0.1% BSA. After 16 hours, 20 μ l of complete medium with IBMX at 3000 μ M (to achieve final concentration of 500 μ M) added to all wells.

Plates were incubated for 10 minutes at 37°C, 5% CO₂ with plates uncovered, before medium was removed and 40 µl of Lysis Buffer 2 was added to all wells. From this point, assays proceeded as described under section entitled “Dose Response”.

2.9.2.3 Time Course

Time course assays were performed similarly to dose response assays, with the exception that all cells were stimulated with peptides at 200 µM in serum-free DMEM with 0.1% BSA and 500 µM IBMX, with time of incubation varied (5-, 10-, 15-, 20-, 30- and 60-minute incubations). An IBMX only control was included for all time points, and results from the corresponding time point normalised to readings from this well.

2.9.2.4 Desensitisation

mHypoA POMC/GFP3 cells were seeded into clear, 96-well plates at 5000 cells per well in complete medium and allowed to adhere overnight. The following day, medium was removed from wells and 40 µl of indicated peptide at 100 nM was added to all wells. Peptide solution was made up without any IBMX. Cells were incubated at 37°C, 5% CO₂ with plates uncovered for 10 minutes before solution was removed from wells, and cells washed three times with pre-warmed HBSS. Cells were then left to recover in 100 µl of serum-free DMEM with 0.1% BSA at 37°C, 5% CO₂ for 1 hour. Medium was then removed from wells and 40 µl of exendin-4 made up in serum-free DMEM with 0.1% BSA and 500 µM IBMX was added to wells at the indicated concentrations, in duplicate. After a 5 minute incubation at 37°C, 5% CO₂ with plates uncovered, 40 µl of Lysis Buffer 2 was added to all wells. Assays then proceeded as described above under “Dose response”.

2.9.3 INS-1 832/3 Cells

The night before the assay, INS-1 832/3 cells growing in T75 flasks were washed twice and placed in low-glucose (2 mM) RPMI medium. The following day, cells were detached with 0.02% EDTA and seeded at 10 000 cells per well in 10 µl of serum-free RPMI medium (11 mM glucose) in opaque half-area 96-well plates (Grenier Bio-One). Cells were stimulated in duplicate with 10 µl of peptide at the indicated concentrations made up in serum-free RPMI medium (11 mM glucose) with IBMX at a final concentration of 500 µM and 0%, 1% or 10% FBS. Cells were incubated for 10 minutes at 37°C, 5% CO₂ with plates uncovered. Assays proceeded from this point onwards as described under “PathHunter CHO-GLP-1R β-arrestin-2 cells”.

2.9.4 MIN6B1 Cells

Six hours in advance of the assay, MIN6B1 cells growing in T25 flasks were washed twice and placed in low glucose (2 mM) RPMI medium. Cells were subsequently detached with 0.02% EDTA and seeded at 10 000 cells per well in 10 µl of serum-free RPMI medium (11 mM glucose) in opaque half-area 96-well plates (Grenier Bio-One). Cells were stimulated in duplicate with 10 µl of peptide at the indicated concentrations made up in RPMI medium (11 mM glucose) with IBMX at a final concentration of 500 µM and 0.1% BSA. Cells were incubated for 10 minutes at 37°C, 5% CO₂ with plates uncovered. Assays proceeded from this point onwards as described under “PathHunter CHO-GLP-1R β-arrestin-2 cells”.

2.10 Measurement of β -arrestin Recruitment

β -arrestin-2 recruitment was determined using the PathHunter β -arrestin assay (DiscoverX). PathHunter CHO-GLP-1R β -arrestin-2 cells in culture were detached with 0.02% EDTA (Sigma) and plated at 10 000 cells per well in 10 μ l of serum-free F12 medium in opaque half-area 96-well plates (Grenier Bio-One). Cells were stimulated with 10 μ l of peptide made up in serum-free F12 medium at the indicated concentration in duplicate. Typically, stimulations for cAMP assays were performed in parallel with the same population of cells. After a 30-minute incubation at 37°C, 5% CO₂ with plates uncovered, 10 μ l of detection reagent was added to all wells (detection reagent made up as per the manufacturer's instructions). Plates were incubated at room temperature in the dark for 60 minutes before luminescence signal was read on a SpectraMax i3x (Molecular Devices) with 500 ms integration time. Data were analysed by applying four-parameter logistic fit in GraphPad Prism 7.03.

2.11 Quantitation of Biased Signalling

Bias between cAMP and β -arrestin-2 was determined by applying a modified form of the operational model of agonism [380]. Concentration response data were fit to the equation below in order to derive a transduction ratio (τ/K_A) value for each agonist in each pathway. The transduction ratio describes the coupling of the agonist to a particular signalling readout in a particular system.

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

Where:

basal = basal level of response of the system

E_{max} = maximum response of the system

τ = measure of agonist efficacy

K_A = pathway specific agonist constant

[A] = molar agonist concentration

n = transducer slope (links receptor occupancy to response)

Basal, E_{max} and n were fit globally given that they are system-specific parameters that are independent of the agonist. Bias was calculated on a per-assay basis, with cAMP and β -arrestin-2 assays being performed in parallel. As τ/K_A is affected by the cell type in which signalling responses are measured, responses were normalised to reference agonist exendin-4. To normalise data, $\log(\tau/K_A)$ values for exendin-4 were subtracted from the $\log(\tau/K_A)$ of each agonist for each pathway, yielding a $\Delta\log(\tau/K_A)$ value. To report bias between the two pathways for each agonist, the $\Delta\log(\tau/K_A)$ value from one pathway (β -arrestin-2 recruitment) was subtracted from the other (cAMP accumulation) to give a log bias factor or $\Delta\Delta\log(\tau/K_A)$. This is summarised below.

Pathway-specific normalisation to exendin-4:

$$\text{Pathway 1: } \Delta\log(\tau/K_A)_{\text{pathway 1}} = \log(\tau/K_A)_{\text{agonist}} - \log(\tau/K_A)_{\text{exendin-4}}$$

$$\text{Pathway 2: } \Delta\log(\tau/K_A)_{\text{pathway 2}} = \log(\tau/K_A)_{\text{agonist}} - \log(\tau/K_A)_{\text{exendin-4}}$$

Reporting bias factor between pathways:

$$\Delta\log(\tau/K_A)_{\text{agonist}} = \Delta\log(\tau/K_A)_{\text{pathway 1}} - \Delta\log(\tau/K_A)_{\text{pathway 2}}$$

As cAMP and β -arrestin-2 data was typically acquired in parallel using the same agonist and cell stock, bias factors were calculated on a per-assay basis.

2.12 Insulin Secretion Assays

Insulin secretion assays were performed using rat pancreatic beta cell line INS-1 823/3 and the Insulin High Range Assay Kit (Cisbio). The night before, cells growing in T75 flasks were washed twice and placed in low-glucose (3 mM) RPMI medium. The following day, cells were detached with 0.02% EDTA and seeded at 10 000 cells per well into 96-well plates in 10 μ l of RPMI medium (11 mM glucose with 1% FBS). 90 μ l of agonist at 10X the target concentration or vehicle (11 mM glucose RPMI medium with 1% FBS) was added immediately and cells were incubated overnight (16 hours) at 37°C, 5% CO₂. After 16 hours, plates were gently agitated on a plate shaker for 5 minutes to achieve uniform distribution of secreted insulin within the well but without detaching cells from the base of the plate. A 10 μ l sample of culture medium was removed and diluted 1:10 in HBSS. Appropriate sample dilutions of supernatant were prepared in HBSS, and added to opaque 96-well, low volume plates. Detection reagents were prepared according to the manufacturer's instructions and added to wells. Plates were incubated at 4 to 24 hours at room temperature, after which HTRF was read on a SpectraMax i3x (Molecular Devices) at wavelengths of 620nm and 665nm.

2.13 DERET Assay for the Measurement of GLP-1R Internalisation

Measurement of GLP-1R internalisation was performed using diffusion-enhanced resonance energy transfer (DERET) [327]. HEK-SNAP-GLP-1R cells in culture were detached with 0.02% EDTA (Sigma) and labelled with 40 nM of Lumi4-Tb in Eppendorfs in 1 ml of complete DMEM without G418 for 60 minutes at room temperature on a HulaMixer Sample Mixer (Thermo Fisher, USA) rotator device. Cells were washed three times with 1 ml of HBSS, before being seeded at 20 000 cells per well in 24 μ M fluorescein in HBSS warmed to 37°C in opaque, half area 96-well plates. Plates were immediately moved to a Flexstation 3 (Molecular Devices) preheated to 37°C, and serial baseline readings taken for 10 minutes by reading time-resolved fluorescence with 340 nm excitation, 520 nm (cut-off 495 nm) and 620 nm (cut off 570 nm) emission, 400 μ s delay time, and 1500 μ s integration time. 10 μ l of peptide was added at 10X the indicated concentration in duplicate, and TR-FRET was read serially every 2 minutes for the following 30 minutes. Net cell surface receptor loss was determined by the ratio of signal obtained at 620 nm to that at 520 nm, after the subtraction of background signal (read from blank wells containing fluorescein but no labelled cells). Data were then normalised to the average basal signal read in the initial 10 minutes, and expressed as fold change/basal.

2.14 TR-FRET Assay for the Measurement of GLP-1R Recycling

CHO-SNAP-GLP-1R cells were seeded overnight at 20 000 cells per well in complete CHO medium without G418 in opaque, half area 96-well plates. On the day of the assay, growth medium was aspirated and surface SNAP-GLP-1Rs were labelled with 40 nM BG-SS-Lumi4-Tb (designed by a collaborator, Dr Johannes Broichhagen, Max Planck Institute for Medical Research, Heidelberg, and

custom synthesised by Cisbio), a cleavable SNAP-Surface probe containing a disulphide bond between O⁶-benzylguanine and Lumi4 moieties, in 20 µl of HBSS + 1% BSA for 30 minutes at 37°C. Cells were then washed three times with 100 µl of HBSS, before stimulation with 50 µl of 100 nM agonist in HBSS for 30 minutes at 37°C, 5% CO₂. Cells were then placed at 4°C and washed with ice-cold HBSS to cause endocytic arrest. Cells were treated with 500 mM ice-cold sodium 2-sulfanylethanesulfonate (MesNa) for 5 minutes, a membrane-impermeable reducing agent, in alkaline TNE buffer (100 mM NaCl, 50 mM Tris-HCl, pH 8.6) to cleave Lumi4-Tb specifically from cell surface receptors. A “minus MesNa” condition for vehicle-treated cells was included to assess the success of receptor labelling. Levels of internalised receptor were measured on a SpectraMax i3x with the following settings: 340 nm excitation, 616 nm emission, 500 µs integration time, 30 µs delay, and 6.28 mm read height. Readings from blank wells (not labelled with BG-SS-Lumi4-Tb) were subtracted from specific signal. Cells were then washed twice with HBSS and incubated with 10 µM LUXendin645 (exendin9-S39C-Cy5, provided by a collaborator, Professor David Hodson, University of Birmingham, described in [381]) in HBSS at 37°C; LUXendin645 acts as both the fluorescence acceptor in TR-FRET measurements, and prevents further receptor internalisation. Plate was immediately transferred to a SpectraMax i3x (Molecular Devices) and time-resolved fluorescence was read in kinetic mode every 2 minutes for 30 minutes with the following settings: 340 nm excitation, 616 nm and 665 nm emission, 30 µs delay and 500 µs integration. The level of recycled receptor was calculated by the ratio of signal obtained at 665 nm to that at 616 nm, after the subtraction of background signal (read from blank wells not labelled with BG-SS-Lumi4-Tb).

2.15 TR-FRET Kinetic Binding Assays

HEK293-SNAP-GLP-1R cells in culture were detached with 0.02% EDTA (Sigma) and labelled with 40 nM of Lumi4-Tb in Eppendorfs in 1 ml of complete DMEM without G418 for 60 minutes at room temperature on a HulaMixer rotator device. Cells were washed three times with 1 ml of HBSS, before being seeded at 30 000 cells per well in 90 µl of HBSS warmed to 37°C with 0.1% BSA and metabolic inhibitors (10 mM NaN₃, 20 mM 2-deoxyglucose, included to prevent GLP-1R internalisation during the subsequent stimulation phases) in opaque, half area 96-well plates. Plates were immediately moved to a Flexstation 3 (Molecular Devices) preheated to 37°C, and serial baseline readings taken for 10 minutes by reading time-resolved fluorescence with 340 nm excitation, 520 nm (cut-off 495 nm) and 620 nm (cut off 570 nm) emission, 50 µs delay time, and 300 µs integration time. Cells were then stimulated with exendin-4-FITC (final concentration 10 nM) with or without unlabelled agonist in duplicate. Plate was immediately returned to the preheated Flexstation 3, and TR-FRET was read serially every 40 seconds for the following 60 minutes with the following settings: 340 nm excitation, 520 nm (cut-off 495 nm) and 620 nm (cut off 570 nm) emission, 50 µs delay time, and 300 µs integration time. Binding was quantified by the ratio of signal obtained at 520 nm to that at 620 nm, after subtraction of the average basal signal read in the initial 10 minutes. Binding data from a dose-response curve of exendin-4-FITC were analysed using the “two or more concentrations of hot” kinetic binding model in Graphpad Prism 7.03 to determine the K_{on} and K_{off} for the labelled ligand on an assay by assay basis. These parameters were then applied for the analysis of binding data for unlabelled compounds to determine kinetic binding constants using the “kinetics of competitive binding” model in Graphpad Prism 7.03.

2.16 Visualisation of GLP-1R Trafficking

2.16.1 mHypoA POMC/GFP3 Cells

mHypoA POMC/GFP3 cells were transiently transfected with N-terminal-EGFP-tagged GLP-1R (provided by a collaborator, Dr Alejandra Tomas, Imperial College London) using Lipofectamine 2000 in 6-well plates as per the manufacturer's instructions. After 24 hours, cells were split and seeded at 50 000 cells per well onto 13 mm circular glass coverslips (Agar Scientific, UK) in 24 well plates in complete DMEM. After a following 24 hours, medium was removed and cells washed twice with HBSS pre-warmed to 37°C. Cells were treated with 100 nM peptide resuspended in pre-warmed HBSS or vehicle (HBSS), for 30 minutes at 37°C, 5% CO₂. After stimulation, cells were moved to ice and washed twice with cold (4°C) PBS, and then fixed with 4% formaldehyde for 20 minutes. Plates were kept covered to minimise light exposure. Cells were rinsed once with cold PBS and mounted using ProLong Diamond Antifade Mountant with DAPI (Invitrogen, USA). Images were acquired on a Zeiss LSM-780 inverted confocal laser-scanning microscope with a 63x/1.4 numerical aperture oil-immersion objective. Images were processed in ImageJ (FIJI).

2.16.2 HEK293-SNAP-GLP-1R Cells

HEK293-SNAP-GLP-1R cells were seeded at 20 000 cells per well onto 13 mm circular glass coverslips (Agar Scientific, UK) in 24 well plates in complete DMEM. After a following 24 hours, medium was removed and cells washed twice with HBSS pre-warmed to 37°C. SNAP-GLP-1R was covalently labelled with 1 µM SNAP-Surface 549 for 30 minutes at 37°C. Cells were then washed with HBSS warmed to 37°C. Cells were then stimulated, and assay proceeded as described above under "mHypoA POMC/GFP3 cells". Images were acquired as described above, or on an ECLIPSE Ti2 inverted microscope with a 40x/0.95 numerical aperture objective. Images were processed in ImageJ.

2.17 RNAseq

mHypoA POMC/GFP3 cells were seeded at 50 000 cells per well in complete DMEM in 12-well plates. After 24 hours, medium was removed and cells were washed twice with PBS pre-warmed to 37°C. Cells were treated with 100 nM peptide resuspended in 2 ml of complete DMEM or vehicle (complete DMEM) for 16 hours at 37°C, 5% CO₂. Medium was removed and cells were washed twice with pre-warmed PBS, before lysis in 350 µl of Buffer RLT with 1% β-mercaptoethanol and sample collection. RNA was extracted using the RNeasy Kit (Qiagen, USA) as per the manufacturer's instructions but with an additional DNase treatment step during the buffer RW1 wash step. Cells were washed with 350 µl of buffer RW1 rather than specified 700 µl, before 80 µl of PureLink DNase (Thermo Fisher, USA; prepared according to the manufacturer's instructions) was added to the spin column membrane. Columns were incubated at room temperature for 15 minutes, and then washed with remained 350 µl of buffer RW1 before resumption of the normal RNeasy protocol.

Further sample processing for RNAseq was performed by collaborators Dr Aida Martinez-Sanchez and Miss Grazia Pizza, Imperial College London. Libraries were analysed by next generation sequencing in the Imperial BRC Genomics Facility. Analysis of differentially expressed genes with each treatment compared to vehicle was performed by Dr Martinez-Sanchez using DESeq2.

2.18 DPP-4 *in vitro* Degradation Assay

DPP-4 degradation assay was performed by Mr Zijian Fang, Imperial College London. 10 nmol exendin-4, exendin-phe1, exendin-asp3 or GLP-1 (positive control) was dissolved in 750 μ l Tris buffer, pH 7.5. 0.3 μ g recombinant DPP-4 enzyme (R&D Systems), or no enzyme as a control for non-enzymatic degradation over the same time period, was added to the reconstituted peptide. Samples were incubated at 37°C and 120 μ l samples were collected from the reaction vessel at 1 and 24 h. 5 μ l 10% trifluoroacetic acid (TFA) was added to each sample to terminate enzyme activity. Samples from degradation assay were analysed by reverse-phase high-performance liquid chromatography (HPLC) with a linear acetonitrile/water gradient acidified with 0.1% TFA on Phenomenex Aeris Peptide 3.6 μ m XB-C18 Column (150 x 4.6 mm). The eluted peptides were detected at 214 nm. Degradation of peptide was calculated by comparing the area under the peak of original compound without and with enzyme.

2.19 Transcardial Perfusion of Mice

Mice were administered fluorescently tagged peptides by intraperitoneal injection at the indicated weight-adjusted dose. After the indicated time, mice were terminally anaesthetised by overdose with 200 mg/kg of sodium pentobarbital (Boehringer Ingelheim Animal Health, UK). As soon as loss of the pedal reflex was observed, a horizontal incision was made just below the xiphoid process to open the abdomen, followed by a longitudinal incision through the right, lateral rib cage from the level of the xiphoid process to the clavicle. The xiphoid process was grasped with forceps and held upwards to expose the diaphragm, and a horizontal incision was then carefully made through the diaphragm. A longitudinal incision was made through the left, lateral rib cage from the level of the xiphoid process to the clavicle to completely expose the chest cavity. A blunt, 21 gauge needle was inserted into the left ventricle at the apex of the heart, and the right atrium was cut with scissors to allow for circulated perfusate to drain. 20 ml of 0.01 M PBS was flushed through the left ventricle, followed by 20 ml of 4% formaldehyde 0.01M PBS solution. Brain and pancreas were immediately dissected and post-fixed for 4 to 24 hours in 4% formaldehyde 0.01M PBS solution at 4°C.

2.20 Tissue Clearing, Imaging and Analysis

Tissue clearing was carried out using the 3DISCO protocol [382]. Brains and pancreata collected after transcardial perfusion were removed from 4% formaldehyde 0.01M PBS solution and washed twice for 1 minute in 0.01M PBS. Sections of pancreata (approximately half of the collected tissue) were embedded in cylindrical (1.5cm diameter, 5cm length), 1% low melting point agarose gels (Invitrogen, USA) made with ultrapure water. Agarose-embedded pancreata and unembedded brains were incubated in glass vials for 12 hours each in the following sequence of solutions (all made in ddH₂O): 50% tetrahydrofuran (THF, Sigma Aldrich), 70% THF, 80% THF, 100% THF, 100% THF, and 100% THF. Tissue was then incubated in 100% dibenzyl ether (Sigma Aldrich) for 16 hours or until tissue was transparent. Glass vials were light protected throughout the process, and all incubation steps were performed on a HulaMixer rotator device. Samples were imaged on a custom-built optical projection tomography (OPT) platform, with a Retiga R1 CCD Camera (Teledyne QImaging, USA), 0.5X magnification telecentric lens (Edmund Optics, UK), excitation filters at 480 $\pm$ 15 nm ("470 nm channel"), 655 $\pm$ 15 nm ("660 nm channel") and 720 $\pm$ 30 nm ("730 nm channel"), and emission filters at 533 $\pm$ 20 nm, 705 $\pm$ 15 nm and 810 $\pm$ 45 nm. Images were acquired from 200 angles, with

integration times of 100 ms, 1000 ms, and 2000 ms for the 470, 660 and 730 nm channels, respectively. Acquisition parameters were kept consistent across samples from the same experiment.

Using custom-written MATLAB scripts, provided by Dr James McGinty, Imperial College London, raw images from the three acquisition channels were reconstructed, cropped and scaled in MATLAB R2016a (MathWorks, USA). To allow for direct comparison of intensity signals between samples, images for all samples from the same channel were scaled upon a shared intensity scale with a global maximum. Scaled images were viewed in 3D in Volocity 6.3.0 (Quorum Technologies, Canada). For brain region quantification, custom-written MATLAB scripts were used to crop out individual regions (hypothalamus, dorsal vagal complex, vascular organ of the lamina terminalis, and subfornical organ) from scaled whole brain images. Images of the cropped region were viewed and quantified in Volocity or ImageJ, by summing the intensity values of all voxels in the region above a threshold intensity of 5000, 7000 or 8000 units. The specific functions used to achieve this were “Find objects” in Volocity, and “3D object counter” in ImageJ. The lower threshold was derived from signal in corresponding regions in vehicle samples from the same experiment, representing tissue autofluorescence. Differences in the lower threshold represent minor differences in tissue autofluorescence levels between experiments. These differences likely result from subtle differences in the clearing process (e.g. differences in the age of the tetrahydrofuran and benzyl ether used) and age of the animals used (as tissue autofluorescence increases with age due to the build-up of autofluorescent substances such as lipofuscin in tissues with a low rate of cell turnover).

For pancreata sections, quantification of signal was conducted in ImageJ (“3D objects counter”) by summing the intensity values of all voxels in the pancreas above a threshold intensity of 2000 units. This lower threshold was derived from autofluorescent signal in vehicle-treated pancreata from the same experiment. Only signal from islet-like structures was included in quantification; large extra-islet structures showing fluorescence were manually identified using the “Objects map” in ImageJ and were excluded from quantification.

2.21 Measurement of Peptide Levels in the Cerebrospinal Fluid

For cerebrospinal fluid (CSF) extractions, the following equipment was prepared. Borosilicate glass capillaries (World Precision Instruments, USA. Dimensions: 1.0 mm outer diameter, 0.58 mm internal diameter, 10 cm length) were pulled on a P-97 Flaming/Brown Micropipette Puller (Sutter Instrument, USA) with the following settings: heat 545, pull 70, velocity 70, time 200. Pulled glass capillaries were inserted into silicone tubing (Instech, USA. Dimensions: 0.76 mm internal diameter, 1.65 mm outer diameter, cut to 30 cm). A blunt 21G needle attached to a 1ml syringe was inserted into the other end of the silicone tubing. Immediately before CSF extraction, the capillary tip was cut with scissors at a 45° angle. Air was drawn into the syringe and then ejected through the syringe-tubing-capillary system into water to check for bubble production and therefore successful opening of the capillary tip.

Rats were administered peptide at the indicated concentration by intraperitoneal injection. At the indicated time point, rats were culled by rising concentrations of CO₂ with permanent cessation of circulation by cutting of the femoral artery as a secondary means of confirmation. Blood was immediately taken by cardiac puncture using heparinized syringes with beveled 21G needles and collected into heparinized Eppendorf tubes prepared with protease inhibitor before being left on ice. Rats were then placed upon a stereotactic frame (Model 900, David Kopf Instruments, USA) using ear

bars in the regular fashion, and incisor bar to hold head in a position such that the dorsal cranial surface formed approximately a 60° angle with the base of the stereotactic frame. Fur was shaved from a rostrocaudal region spanning from between the ears to the top of the scapula. A 3 cm longitudinal midline incision was made from between the ears to the top of the scapula and skin pulled apart to expose the superficial muscle layers. Muscle layers were carefully separated down the midline using a scalpel to expose the base of the skull and the atlas bone. The atlanto-occipital membrane between the base of the skull and the atlas bone was pierced with a sharpened glass capillary at approximately a 45° angle from the dorsal surface. If pierced correctly (indicated by CSF immediately flowing into the capillary), the angle was increased to approximately 60° to aid CSF flow. If CSF flow ceased before adequate volume collection (100 µl), syringe was gently retracted to draw CSF into the capillary. Collected CSF was ejected into an Eppendorf and snap frozen in liquid nitrogen. Plasma was separated from collected peripheral blood by centrifugation for 8 minutes at 10 000 g, 4°C. CSF and blood exendin-4 and exendin(9-39) concentration was measured by an exendin-4 ELISA (Phoenix Pharmaceuticals, USA) according to the manufacturer's instructions.

2.22 Ca²⁺ imaging in *ex vivo* brain slices of Glp1r-Cre/GCaMP3 mice

Glp1r-Cre/GCaMP3 mice (i.e. mice in which *Glp1r* promoter-driven Cre recombinase expression leads to expression of the Ca²⁺ reporter protein GCaMP3 specifically in GLP-1R-expressing cells) were deeply anaesthetised using isoflurane and decapitated. The forebrain was removed and placed in ice-cold high Mg²⁺/low Ca²⁺ artificial cerebrospinal fluid (ACSF; 2.5 mM KCl, 200 mM sucrose, 28 mM NaHCO₃, 1.25 NaH₂PO₄, 7 mM glucose, 7 mM MgCl₂, 0.5 mM CaCl₂; pH 7.4). 200 µm-thick coronal slices of the hypothalamus containing the ARC were cut on a vibratome (Model 7000smz2, Campden Instruments, UK); anatomical localisation was conducted by comparison to The Mouse Brain in Stereotaxic Coordinates [383]. For all experiments, slices were taken from locations between 1.46 mm and 2.06 mm caudal from bregma. Slices were incubated in recovery solution (3 mM KCl, 118 mM NaCl, 25 mM NaHCO₃, 1.2 NaH₂PO₄, 2.5 mM glucose, 7 mM MgCl₂, 2 mM CaCl₂; pH 7.4) at 34°C for a minimum of 1 hour. All solutions were constantly bubbled with 95% O₂/5% CO₂.

Ca²⁺ imaging was performed on *Glp1r*-expressing neurons in the ARC using a Zeiss Axioskop 2 FS widefield microscope using a 40x water immersion lens. GCaMP3 was excited using a LED light source (CoolLED pE300white; QImaging) at 460 nm (± 25 nm) for 250 ms every 5 s. Images were captured at 12-bit on a charge-coupled device camera (Q-Click; QImaging). Tissue slices were constantly superfused with 30°C standard ACSF standard ACSF (3 mM KCl, 118 mM NaCl, 25 mM NaHCO₃, 2 mM glucose, 8 mM mannitol, 1 mM MgCl₂, 2 mM CaCl₂; pH 7.4) at a flow rate of 4 ml/min. All treatments were made up in 30°C standard ACSF and superfused at the same rate. Slices were treated with 10 nM of the indicated peptide for 10 minutes, followed by a 10 minute wash with ACSF, then 100 µM glutamate for 1 minute to test for the maintenance of neuronal responsivity, followed by a final 10 minute wash with ACSF. Tissue slices were then allowed to recover for 2 hours in recovery solution bubbled with 95% O₂/5% CO₂. Tissue slices then underwent re-challenge with 100 nM of exendin-4 for 10 minutes, followed by a 10 minute wash with ACSF, 1 minute 100 µM glutamate treatment, then a final 5 minute wash with ACSF.

Data was collected from four different experiments using tissue derived from a different mouse in each experiment. Recordings were analysed using ImageJ. XY-drift was corrected for using the "StackReg" plugin where possible, and conducted manually where not. Regions of interest (ROIs) and

an area for determining background fluorescent intensity were chosen and the mean pixel intensity calculated for each region, for each time point. Background intensity at the corresponding time point was subtracted from the measurements for each ROI to obtain “background-corrected” values. Recordings were adjusted for photobleaching using a cubic polynomial function on OriginPro (OriginLab Corporation, USA). For individual ROI recordings, four points at the following time points were selected to draw the cubic polynomial function: at the onset of recording, at the start of peptide stimulus, just before glutamate treatment, and in the middle of the final ACSF wash. The resulting cubic polynomial function was used as a time-dependent “baseline”. Baseline values from the corresponding time point were subtracted from background-corrected values for each ROI to determine the corrected fluorescence intensity, “ ΔF ”. ΔF values were then normalized to F_0 , the average fluorescence intensity in the 5 minutes prior to peptide stimulus. Fluorescence intensity data are therefore reported as $\Delta F/F_0$. Responses to peptides were quantified by calculating the area under the curve of $\Delta F/F_0$ values during the 10 minute stimulus.

Glp1r-Cre/GCaMP3 mice were kindly provided by Professor Stefan Trapp, University College London. Experiments were conducted in the Trapp laboratory by Wanqing Jiang with the assistance of the author. Data analysis was shared between Wanqing Jiang and the author.

2.23 Statistical Analyses

All data analyses were performed using Graphpad Prism 7.03. (Graphpad Software, USA). Data is expressed throughout as mean $\pm$ SEM unless otherwise stated, and with individual replicates shown where appropriate. One- or two-way ANOVAs were performed throughout, the latter being used for experiments with multiple time-points. *Post hoc* tests were applied based upon the primary experimental question. Tukey's test was applied to assess differences between all groups, and Dunnett's test where specific comparisons with exendin-4 or vehicle were being made. Repeat-measures (randomised block) ANOVAs were performed if experiments were conducted with a matched design. For *in vitro* assays, the intra-experimental replicate mean was treated as a single experimental replicate. Curve fitting and bias calculations were performed as described in sections above. Two exceptions to the parametric tests primarily used were for analysis of Glp1r-Cre/GCaMP3 brain slice Ca^{2+} responses and RNAseq experiments. Mann-Whitney U tests, and Wald tests with Benjamini-Hochberg adjustment, respectively, were used for analysis of these experiments.

3 The Physiological Effects of Biased GLP-1RAs after Peripheral Administration

3.1 Introduction

The existence of biased agonism at the GLP-1R is well established and there has been significant progress towards understanding its structural basis [374]. Moreover, recent studies have demonstrated its potential therapeutic utility in optimising anti-diabetic medications [323], [370]. Multiple or single amino acid changes to the N-terminus of the exendin-4 sequence produces agonists biased towards G-protein signalling (“G-protein biased”) or β -arrestin recruitment (“ β -arrestin biased”), with G-protein biased agonists exhibiting enhanced anti-hyperglycaemic effects *in vivo* [323], [370]. The findings of the more recent of these two studies forms the foundation of the following investigation, and are detailed below.

3.1.1 G-protein-biased vs. β -arrestin-biased exendin-4 analogues

Given the importance of the agonist N-terminus in GLP-1R activation, Jones et al. synthesised a panel of exendin-4 analogues with single amino acid substitutions in this region [323]. The propensity of each analogue to induce cAMP accumulation and β -arrestin recruitment was assessed, and signal bias calculated relative to parent compound exendin-4. Additionally, agonist-induced GLP-1R internalisation and insulin secretion in pancreatic β -cell lines INS-1 823/3 (rat-derived) and MIN6B1 (mouse-derived) after prolonged, 16-hour stimulation (to mimic *in vivo* drug exposure) was characterised for the analogues. G-protein-biased agonists consistently showed lower levels of receptor internalisation and superior insulinotropism, whereas β -arrestin-biased compounds exhibited the opposite characteristics. These findings are in keeping with the canonical role of β -arrestins in receptor desensitisation and internalisation and suggest that the reduced β -arrestin recruitment of G-protein-biased GLP-1R agonists facilitates prolonged signalling.

3.1.1.1 *In vitro* characteristics of exendin-phe1 and exendin-asp3

Of the panel of analogues, the *in vitro* properties of a single G-protein-biased agonist – “exendin-phe1”, featuring a phenylalanine substitution at position 1 – and a single β -arrestin-biased agonist – “exendin-asp3”, featuring an aspartic acid substitution at position 3 – were subjected to further examination. In addition to reduced levels of overall receptor internalisation, exendin-phe1 treatment resulted in a significantly faster rate of receptor recycling to the plasma membrane, and reduced levels of lysosomal degradation. Exendin-asp3, by contrast, showed a reduced rate of receptor recycling and a modestly increased overall level of receptor internalisation relative to exendin-4, though comparable levels of degradation.

In keeping with these trafficking profiles, cAMP signalling in pancreatic β -cell lines after prolonged incubation (3 or 24 hours) was significantly elevated for exendin-phe1 relative to exendin-4 (despite being less potent after an acute, 10-minute stimulation), whereas it was significantly reduced for exendin-asp3. Consistent with the broader analogue panel and the demonstrated link between G_s signalling and insulin secretion, exendin-phe1 and exendin-asp3 resulted in significantly greater and lower levels of insulin secretion in pancreatic β -cells *versus* exendin-4, respectively. Notably, these differences were time-dependent, only emerging after 3 hours of stimulation, and becoming maximal at 16 hours. Thus, whilst being a slightly less potent GLP-1R agonist acutely, the overall trafficking phenotype of G-protein-biased exendin-phe1 results in the maintenance of a larger pool of sensitised receptors at the plasma over time at steady state, allowing time-dependent signalling and

insulinotropic advantages to emerge. In opposition, β -arrestin-biased exendin-asp3 leads to greater GLP-1R desensitisation and internalisation, leading to diminished maximal signalling and secretory responses after prolonged stimulation.

3.1.1.2 *In vivo characteristics of exendin-phe1*

The physiological impacts of GLP-1R G-protein bias were investigated for exendin-phe1. Consistent with its enhanced insulinotropism *in vitro*, exendin-phe1 conferred superior anti-hyperglycaemic effects *versus* exendin-4 in IPGTTs in diet-induced obese (DIO) mice. As with *in vitro* observations, this effect was time-dependent. When agonist and glucose challenge occurred concomitantly, exendin-phe1 and exendin-4 performed similarly. By contrast, when a delay was included between agonist administration and glucose challenge to allow the trafficking-related advantages of exendin-phe1 to emerge, exendin-phe1 induced greater anti-hyperglycaemic effects associated with increased insulin release. These differences were significant with a 4-hour delay, and further increased with an 8-hour delay. Importantly, the two agonists had matched pharmacokinetic profiles, excluding the possibility that their differential glycaemic effects were due to differences in plasma concentration. Moreover, after chronic, 10-day administration in DIO mice, exendin-phe1 continued to exert anti-hyperglycaemic effects when assessed by IPGTT, whereas exendin-4 failed to do so. Again, plasma concentrations were matched.

Despite their markedly different glycaemic effects, the anorectic and weight-lowering effects of exendin-4 and exendin-phe1 were identical in both acute and chronic settings. However, a rodent correlate of nausea – pica, the ingestion of non-nutritive materials – was observed less frequently in exendin-phe1-treated animals. Exendin-phe1's improved glycaemic effects may therefore come with the added benefit of reduced nausea, the main side effect of pharmacological GLP-1RAs and a major reason for treatment discontinuation [384]. Thus, exendin-phe1's selective enhancement of β -cell effects may pose it as more efficacious and more tolerable therapeutic relative to exendin-4.

The difference in the relative effects of exendin-4 and exendin-phe1 in the contexts of glucoregulation and appetite suppression suggests that a divergence exists between the manifestations of GLP-1R biased signalling between the two physiological systems. Exendin-phe1's *in vitro* biased signalling and related trafficking profile is paralleled in its glycaemic effects, though any signalling advantage relative to exendin-4 is lost when anorectic effects are examined. Whether these findings are generalisable to β -arrestin-biased agonists is yet unexplored.

The following chapter aims to broaden the previous physiological investigation by including β -arrestin-biased exendin-asp3 in its studies and quantitatively establishing the divergence between their physiological effects. As before, anorectic and glycaemic responses to the agonists will be examined, given their tractability and that fact that they are the two major, relevant metabolic effects of pharmacological GLP-1RAs in the treatment of T2DM. Based upon its *in vitro* properties, it is predicted that exendin-asp3 will demonstrate weaker anti-hyperglycaemic effects in delayed IPGTTs relative to exendin-4. Whilst exendin-asp3's β -arrestin-biased profile may also predict weaker anorectic effects, a similar loss of signal bias manifestation seen for exendin-phe1 may also be observed for exendin-asp3. If seen, this would corroborate the previously observed divergence between the glucoregulatory and anorectic effects of biased exendin-4 analogues.

For convenience, exendin-4, G-protein-biased exendin-phe1 and β -arrestin-biased exendin-asp3 will hereafter be referred to as “biased GLP-1RAs” when being discussed collectively.

3.1.2 Aims

1. To examine the glycaemic responses to biased GLP-1RAs after peripheral administration.
2. To examine the anorectic effects of biased GLP-1RAs after peripheral administration.
3. To quantitatively establish the divergence between these two responses.

3.2 Results

3.2.1 OGTT and IPGTT glycaemic response after peripheral administration of biased GLP-1RAs

IPGTTs were conducted after peripheral administration of exendin-4, exendin-phe1, and exendin-asp3, with either no delay between peptide administration and glucose challenge (“acute”), or with glucose challenge occurring 6 hours after peptide administration (“6h delay”). When administered acutely, all agonists exhibited equal and potent anti-hyperglycaemic effects (Figure 3.1 A). By contrast, with a 6 hour delay, glycaemic responses to the agonists differed markedly: exendin-phe1 continued to show potent anti-hyperglycaemic effects, exendin-4 induced moderate anti-hyperglycaemic effects, and exendin-asp3 performed even worse than vehicle (Figure 3.1 B). Similar results were obtained with a 6 hour delay in an oral glucose tolerance test (OGTT, Figure 3.1 C). Insulin readings were not taken in these experiments, thereby precluding direct links between agonist action and insulin secretion. However, it has been previously shown that at least for exendin-4 *versus* exendin-phe1, glucoregulatory differences are associated with greater insulin release with the latter [323].

The data featured here are consistent with *in vitro* observations, where agonist-associated differences in cAMP accumulation and insulin secretion only emerge after several hours of exposure in pancreatic β -cell lines INS-1 832/3 and MIN6B1 [323]. Given the lack of pharmacokinetic differences between the agonists (see Section 3.2.2 and [323]), the opposing trafficking phenotypes of exendin-phe1 and exendin-asp3 that underlie these *in vitro* observations likely form the basis of the differential glycaemic responses seen *in vivo*.

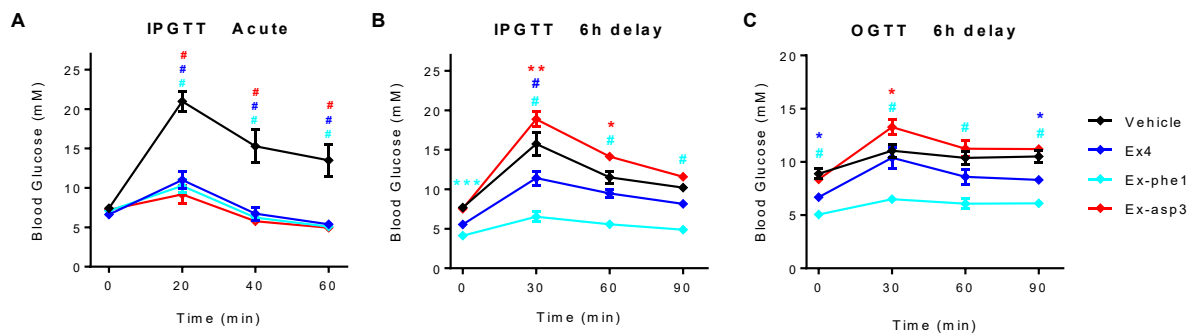


Figure 3.1. Glycaemic responses after peripheral administration of biased GLP-1RAs.

(A) Blood glucose during IPGTT (2 g/kg glucose) in lean, male C57Bl/6 mice after intraperitoneal agonist injection (3 nmol/kg), n=8/group except vehicle (n=7). (B) As for (A) but with a 6 hour delay between agonist administration and glucose challenge, n=8. (C) Blood glucose during OGTT in lean, male C57Bl/6 mice after oral gavage of glucose (2 g/kg) and 6 hours after intraperitoneal agonist administration (3 nmol/kg), n=8/group except vehicle (n=7). Two-way ANOVA with Tukey’s test, with significance vs. saline. *p<0.05, **p<0.01, #p<0.0001. Data represented as mean $\pm$ SEM.

On a technical note, OGTTs are a more physiological assessment of glucose handling than IPGTTs as they take into account the incretin effect and gastric emptying time [385]. Conversely, these additional factors confound a direct assessment of agonists upon β -cell insulin secretion. Because of

this, and the technical ease of conducting an IPGTT, all subsequent GTTs conducted in this project are IPGTTs.

3.2.2 Measurement of plasma levels of biased GLP-1RAs

An alternative explanation for exendin-phe1's enhanced, and exendin-asp3's worsened anti-hyperglycaemic effects after a delay could be pharmacokinetic differences – that is, the former has a longer, and the latter a shorter circulating half-life than exendin-4, leading to differences in plasma levels that would explain the differences in duration of anti-hyperglycaemic action seen above. An alanine to glycine substitution at position 2 underlies exendin-4's resistance to DPP-4 degradation relative to GLP-1, as DPP-4 preferentially cleaves N-terminal X-Pro or X-Ala dipeptide sequences [386]–[388]. Therefore, it is feasible that the N-terminal phenylalanine and aspartic acid substitutions of exendin-phe1 and exendin-asp3 may also lead to changes in susceptibility to degradation. To test this, mice were administered 100 nmol/kg of exendin-4, exendin-phe1 or exendin-asp3, and blood collected 6 hours later to correspond with the delayed GTT paradigm. In order to remain within assay detection limits, a high dose was administered given the relative rapidity of exendin-4's plasma clearance. An ELISA that recognises the C-terminus of exendin-4 was used to measure plasma levels given that the C-terminus is identical for the three peptides. Importantly, assay recovery of the three peptides was matched (Figure 3.2 A). No difference in plasma levels of the agonists was observed at 6 hours (Figure 3.2 B), suggesting that pharmacokinetic differences do not account for the differences in glucoregulatory effects seen in delayed GTTs. Moreover, no *in vitro* degradation by DPP-4 over 24 hours with any of the exendin analogues was detected by HPLC analysis (Figure 3.2 C; assay performed by Mr Zijian Fang, Imperial College London).

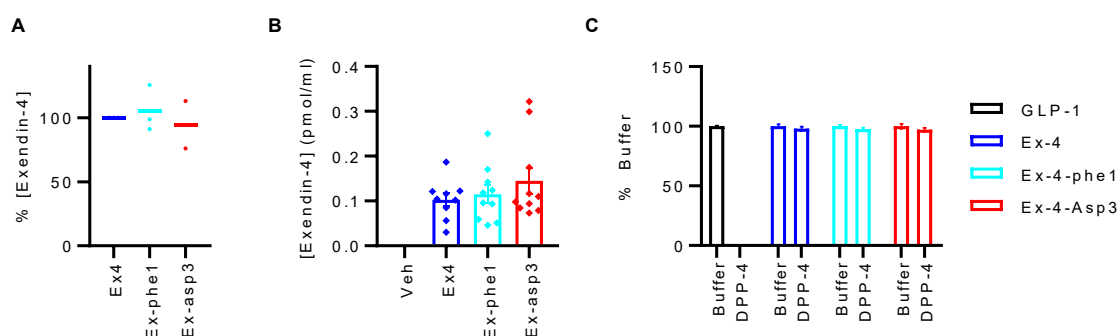


Figure 3.2. Measurement of plasma levels of biased GLP-1RAs.

(A) Measured exendin-4 levels in ELISA buffer spiked with exendin-4, exendin-phe1 or exendin-asp3, and expressed after normalisation to exendin-4 values, $n=2-3$. **(B)** Plasma exendin-4 concentration 6 hours after intraperitoneal administration of 100 nmol/kg of exendin-4, exendin-phe1 or exendin-asp3, $n=9-10/\text{group}$. One-way ANOVA with Tukey's test. **(C)** Peptide levels measured by HPLC after incubation for 24 hours with ("DPP-4") or without ("Buffer") DPP-4 *in vitro*. Peptide levels in DPP-4-treated conditions are expressed as a percentage of those in untreated conditions for the same peptide, $n=3$. All data represented as mean $\pm$ SEM.

3.2.3 Anorectic responses after peripheral administration of biased GLP-1RAs

As GLP-1RAs promote satiety and reduce food intake, anorectic responses after peripheral administration of biased GLP-1RAs were assessed in both male, lean C57Bl/6 mice and male, lean Wistar rats to investigate whether differences in agonist bias were also manifested in the context of appetite regulation. Rodents were fasted during the dark phase before their diet was returned at the start of the light phase after intraperitoneal administration of vehicle, exendin-4, exendin-phe1 or exendin-asp3 at either 0.1, 0.3, 1, 2, 3 or 10 nmol/kg. As the manifestation of bias-related differences between the tested agonists is time-dependent, dose response curves for cumulative food intake reduction at the assessed time points were constructed in order to examine time-related effects more clearly (Figure 3.3 A, C). Cumulative food intake over time for two key doses (1 and 3 nmol/kg) are also displayed (Figure 3.3 B, D). What is immediately evident from these graphs is a lack of large, consistent difference in the anorectic effects of the agonists, despite clear differences in glycaemic responses seen above (Figure 3.1 B, C). Thus, although bias-related differences are manifested in the context of glucoregulation, this does not translate into commensurate effects on appetite regulation when agonists are administered peripherally.

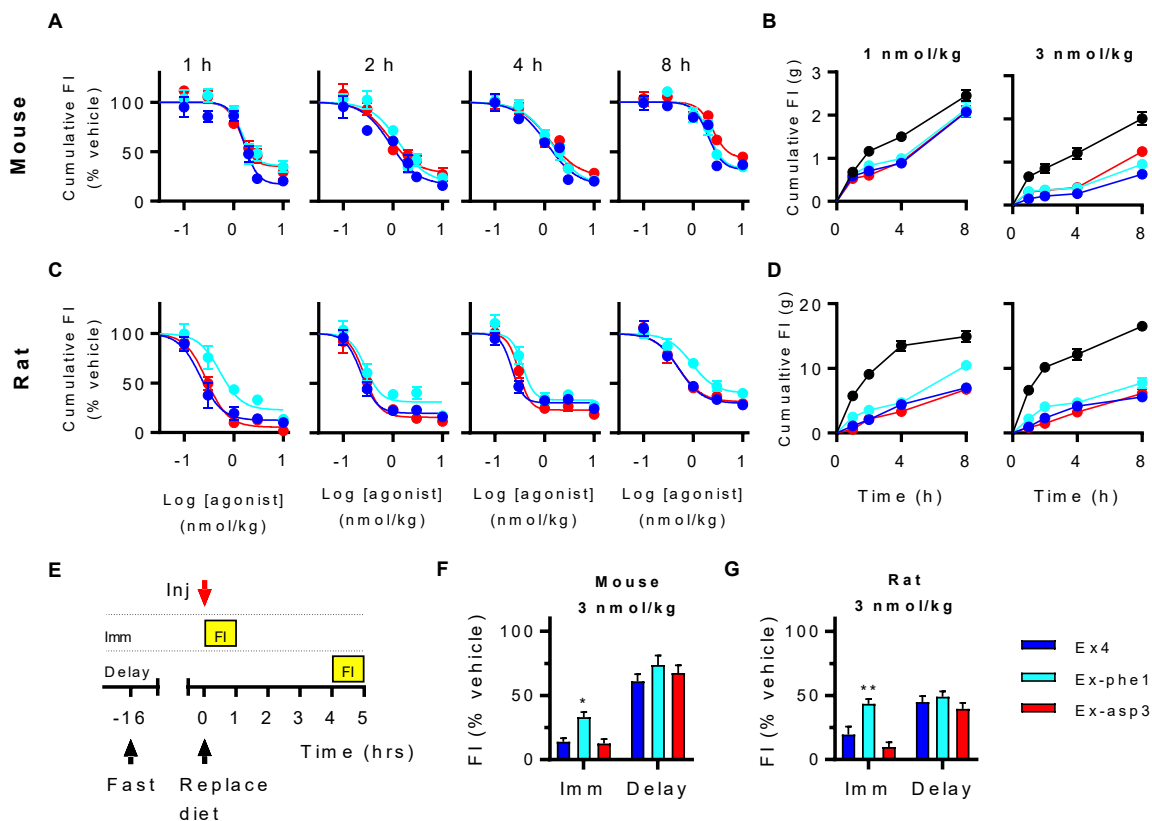


Figure 3.3. Anorectic responses after peripheral administration of biased GLP-1RAs in rodents.

(A) Cumulative food intake at the indicated time point in male, lean C57Bl/6 mice, expressed relative to food intake from the corresponding vehicle group, with 4-parameter logistic fit of the data. Agonists administered by intraperitoneal injection at 0.1, 0.3, 1, 2, 3 and 10 nmol/kg, $n=8$ /group except vehicle ($n=7$). **(B)** Cumulative food intake over time at the indicated dose for mice, $n=8$ /group except vehicle ($n=7$). **(C-D)** As for (A) and (B) but in male, lean Wistar rats, $n=8$. **(E)** Diagram explaining food intake paradigm used in (F) and (G), with diet being replaced either immediately ("Imm"), or 4 hours ("Delay") after agonist administration. **(F)** Food intake expressed relative to vehicle in the first hour following replacement of diet in mice. Agonist administered at 3 nmol/kg, $n=8$ /group except vehicle ($n=7$). **(G)** As for (F) but in rats, $n=8$. Two-way ANOVA with Dunnett's test vs. exendin-4 response. * $p<0.05$, ** $p<0.01$. Data represented as mean $\pm$ SEM.

A subtle time-related effect was observed however, with exendin-phe1 being less anorectic in rats at the 1-hour time point than exendin-4, with the difference tending to disappear by 4 hours, but then re-emerging at 8 hours. This effect is evident in rats at all tested doses – as indicated by the rightward shift of the dose response curve at 1 hour and 8 hours (Figure 3.3 C). This pattern might be rationalised by the reduced acute potency of exendin-phe1 leading to a reduced acute anorectic effect, but with relative efficacy advantages over the subsequent 1-3 hours due to reduced desensitisation, followed eventually by loss of effect at the final time-point as the drug concentration falls below the threshold at which this lower-potency compound is active. A somewhat similar pattern, but less marked and only at doses above 3 nmol/kg, was observed in mice. This species-related effect may be explicable by differences in agonist efficacy at the rat *versus* mouse GLP-1R. The magnitude of difference in cAMP accumulation and insulin secretion between exendin-4 and exendin-phe1 are smaller in mouse pancreatic β -cell line MIN6B1 as compared to rat pancreatic β -cell line INS-1 832/3 [323]. Higher doses of agonist may therefore be required in mice *versus* rats in order to observe exendin-phe1's acute deficit in anorectic efficacy. As a further subtle observation, in mice, some of the higher doses of exendin-asp3 appeared to “wear off” slightly faster than for exendin-4, leading to a slight right shift in the cumulative dose response curve at 8 hours (Figure 3.3 A).

To further investigate this time-related effect, food intake assays were conducted in which diet was either returned immediately after peptide administration at 3 nmol/kg (as per the normal experimental design), or 4 hours after peptide administration, with food intake assessed over the subsequent hour (Figure 3.3 E). Consistent with dose response experiments, exendin-phe1 was significantly less anorectic when food was returned immediately, though performed comparably after a 4 hour delay (Figure 3.3 F, G). Thus, the anorectic effects of exendin-phe1 appear to have a delayed onset relative to those of exendin-4 and exendin-asp3.

3.2.4 Quantification of bias between glucoregulatory and anorectic effects of biased GLP-1RAs

The results above reveal a clear divergence in the physiological manifestations of biased signalling at the GLP-1R. *In vitro*, quantification of biased signalling between two intracellular pathways is achieved by comparing the relative potency and efficacy (or a composite measure that takes account of both) of a test agonist to that of a reference agonist for the two pathways. This generates a “bias factor” that reports the extent of difference in relative agonist activity between the two pathways; the higher the value, the more “biased” the agonist. Dose response curves from which potency and efficacy values are derived are easily produced from *in vitro* assays given their miniaturised and high-throughput nature. Though more labour-intensive, dose response curves could also be constructed for *in vivo* responses, and bias subsequently quantified between the two physiological readouts. Bohn and colleagues take a similar approach with μ -opioid receptor agonists, generating dose response curves for, and comparing bias between the undesirable effect of respiratory depression (quantified as oxygen saturation) with the desired effect of analgesia (quantified by latency to response in standard nociceptive hot plate or tail flick tests) [389]. This allowed direct correlation of bias factor between G-protein signalling and β -arrestin-2 recruitment *in vitro* with that between analgesia and respiratory depression *in vivo* (functionally reported as a wider therapeutic window).

Here, dose response curves were constructed for glucoregulatory and anorectic responses (performed separately and with a different experimental approach to the results shown in Figure 3.3)

to the biased GLP-1RAs. IPGTTs were conducted either acutely or with a 6 hour delay after administration of agonists at 0.01, 0.1, 0.3, 1, 3 or 10 nmol/kg, and area under the curve calculated to create dose response curves (Figure 3.4 A-E). Acutely, no difference in effect was observed between the agonists for doses above 0.3 nmol/kg, though at the lower doses of 0.1 and 0.01 nmol/kg exendin-phe1 induced a greater anti-hyperglycaemic effect (Figure 3.4 E). This is somewhat unexpected given the reduced potency of exendin-phe1 at the GLP-1R for cAMP accumulation, and the lack of difference in insulin secretion between the agonists after short (1 hour) exposure in pancreatic β -cell lines. Interestingly, Zhang *et al.* obtained similar results with an exendin-4-based, G-protein biased GLP-1R agonist ("P5") in acute GTTs: P5 showed improved anti-hyperglycaemic effects relative to exendin-4 when dosed at 0.1 μ g/kg ($\sim$ 0.02 nmol/kg) and 1 μ g/kg ($\sim$ 0.2 nmol/kg) in lean mice, but not at 10 μ g/kg ($\sim$ 2 nmol/kg) [370]. Constructing dose response curves using blood glucose readings from the 20- and 60-minute time points of the acute GTT shows that this beneficial effect of exendin-phe1 was present at the latter but not former time point (Figure 3.4 D). This pattern appears to be in keeping with the timescale over which trafficking differences are manifest.

After a 6 hour delay and in keeping with all previous data, exendin-phe1 showed a clear advantage relative to the other two agonists, as indicated by the leftward shift of its dose response curve (Figure 3.4 E). However, unlike the initial IPGTT and OGTTs presented in this chapter (Figure 3.1), the modest differences between exendin-asp3 and exendin-4 after a 6 hour delay were not replicated, or at least were reduced. For example, for the equivalent dose (3 nmol/kg), exendin-asp3 showed a clear anti-hyperglycaemic effect in Figure 3.4, whereas in Figure 3.1 glucose tolerance was in fact worse than vehicle with exendin-asp3. The reasons for this difference are unclear. Mice were fasted for an equivalent duration in all studies, studies were conducted at a similar time of day, and blood glucose readings in vehicle-treated mice show a similar peak ($\sim$ 15.5 mM) suggesting comparable metabolic profiles for the cohorts (given the potential for age-related decline in pancreatic β -cell function). Nevertheless, the most important difference, i.e. the advantage of exendin-phe1 *versus* the two ligands with higher β -arrestin recruitment, was clearly evident.

Similarly, the acute (first hour) anorectic effect of the agonists were tested at 0.01, 0.1, 0.3, 1, 3, 10 and 100 nmol/kg either immediately or 6 hours after administration with ongoing fasting, and dose response curves constructed. In keeping with results above (Figure 3.3 A), only subtle differences were observed between the agonists in mice (Figure 3.4 F, Table 3.1). Acutely, exendin-phe1 was minorly right-shifted, indicating slightly reduced potency. After a 6 hour delay, both exendin-phe1 and, supporting the subtle effect shown at later time-points in Figure 3.3 A, exendin-asp3, were right-shifted relative to exendin-4, with exendin-phe1 being more affected.

Quantification of bias between anti-hyperglycaemic and anorectic effects at the two time points (Figure 3.4 G) report exendin-phe1 to be biased towards anti-hyperglycaemic effects by a factor of 5 relative to exendin-4 in an acute setting, with the magnitude of its bias increasing to 36-fold after a 6 hour delay. Exendin-asp3 shows weak, time-dependent bias: acutely, it is biased by a 1.7-fold factor toward anorectic effects whereas it is oppositely biased by a factor of 1.6-fold after a 6 hour delay. Although these overall trends can be determined visually by comparison of the dose response curves, formal quantification provides an accessible summary of the data and allows more definitive statements about the magnitude of bias between compounds to be made.

Acute appetite suppression after agonist administration may be used as an indicator of treatment-induced nausea. Taking this interpretation of the anorectic effects, these results can be alternatively expressed as a therapeutic window by taking the ratio between the ED_{50} values for glycaemic and anorectic responses, as has been previously conducted [389]. A larger value indicates greater separation between the two responses i.e. stronger anti-hyperglycaemic effects with reduced nausea. Although absolute values are only relevant within the conducted experiment, it is clear G-protein-biased exendin-phe1 has a much wider therapeutic window than neutral exendin-4 or β -arrestin-biased exendin-asp3 (Figure 3.4 H). This is evident acutely after agonist administration, and increases further after a 6 hour delay. Thus, in keeping with past reports [323], exendin-phe1 confers superior anti-hyperglycaemic effects with diminished correlates of nausea.

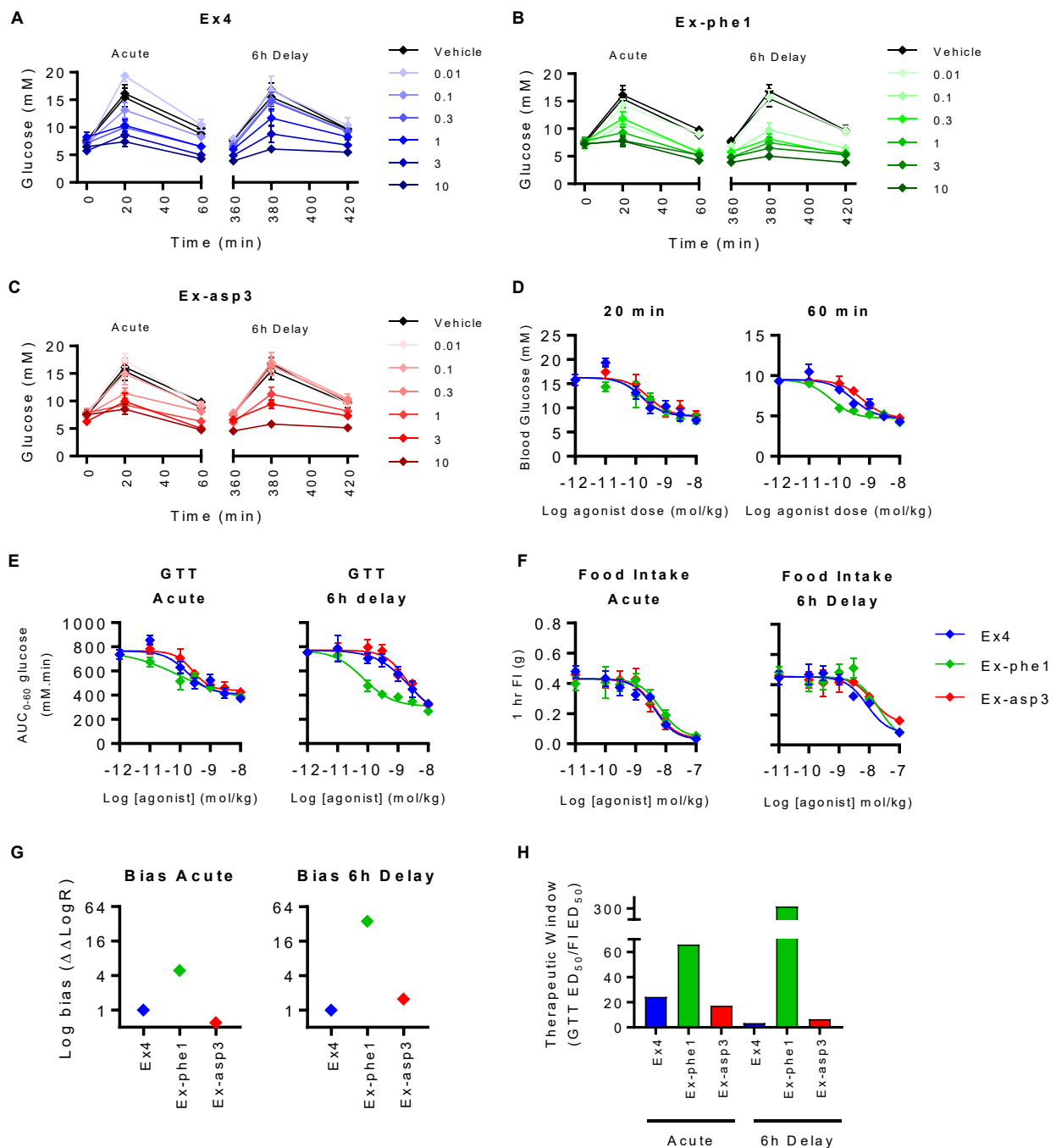


Figure 3.4. Glycaemic and anorectic dose responses after peripheral administration of biased GLP-1Rs in lean mice.

(A-C) Blood glucose during IPGTT (2 g/kg glucose) in lean, male C57Bl/6 mice immediately and 6 hours after intraperitoneal agonist injection (0.01, 0.1, 0.3, 1, 3, and 10 nmol/kg), n=8/group. **(D)** Dose response curves constructed from blood glucose readings at 20- and 60-minute time points of the acute GTT in (A-C) with 4-parameter logistic fit of the data. **(E)** Dose response curves constructed from AUC for blood glucose from (A-C) with 4-parameter logistic fit of the data. **(F)** Dose response curves for food intake reduction over the first hour in lean, male C57Bl/6 mice either immediately or 6 hours after intraperitoneal agonist injection (0.01, 0.1, 0.3, 1, 3, 10 and 100 nmol/kg), n=6-8/group. 4-parameter logistic fit of the data. **(G)** Quantitation of bias for data in (E) and (F) with $\log(\tau/K_A)$ values for each agonist normalised to reference agonist exendin-4. **(H)** Therapeutic window for the biased GLP-1RAs determined by the ratio of ED_{50} values of GTT and food intake data in Table 3.1. Data represented as mean $\pm$ SEM.

		GTT pED ₅₀	Food Intake pED ₅₀
Acute	Exendin-4	9.76	8.37
	Exendin-phe1	10.0	8.18
	Exendin-asp3	9.56	8.35
6 hour delay	Exendin-4	8.62	8.08
	Exendin-phe1	10.2	7.67
	Exendin-asp3	8.72	7.90

Table 3.1. pED₅₀ values for glycaemic and anorectic effects of the biased GLP-1RAs.

pED₅₀ values of the biased GLP-1RAs for glycaemic and anorectic effects as derived from dose response curves featured in Figure 3.4 E & F.

3.3 Discussion

The results presented in this chapter confirm the existence of a divergence between the physiological manifestations of biased GLP-1R signalling in the contexts of glucoregulation and appetite control. They replicate the previous finding that G-protein-biased exendin-phe1 exhibits enhanced anti-hyperglycaemic effects without commensurate increases in anorectic efficacy [323]. Moreover, by including β -arrestin-biased exendin-asp3, they evidence that the physiological divergence extends to oppositely biased GLP-1R agonists. As predicted by its trafficking profile and its related time-dependent deficits in insulinotropism *in vitro* [323], exendin-asp3 displayed comparable glucoregulatory effects in an acute GTT, though worsened effects relative to exendin-4 and exendin-phe1 after a 6 hour delay. This did not, however, come with significant decreases in anorectic efficacy, which were at most slightly reduced. Thus, despite clear time-dependent differences in glycaemic responses between the three agonists, anorectic responses are largely equivalent – that is, the manifestations of biased GLP-1R signalling appear to be “lost” in the context of appetite regulation.

Two possible explanations for the observed divergence between the glycaemic and anorectic effects of the biased GLP-1RAs are:

1. Differences exist in the ability of the agonists to access CNS-located appetite regulatory centres where they exert their anorectic effects; or
2. Differences exist between the direct effects of biased GLP-1RAs upon neurons compared to pancreatic β -cells (i.e. *tissue-specific signalling* responses exist).

Regarding the former, the BBB prevents the free passage of circulating factors into the CNS and thus, for many substances, uptake occurs in a regulated transporter- or receptor-mediated manner [390]. For example, leptin entry into the brain and access to the ARC relies upon LepR endocytosis in tanycytes: specialised, glial/ependymal cells of the blood brain barrier [391]. Leptin-leptin receptor complex is subsequently transcytosed across the cell and released into the cerebrospinal fluid of the 3rd ventricle, facilitating its access to ARC, and possibly extra-hypothalamic LepRs. A similar, endocytosis-dependent mechanism may exist for GLP-1R ligands. In support of this notion, brain uptake of liraglutide is absent in *Glp-1r* knockout mice [179].

If a GLP-1R-dependent mechanism is extant, differences in agonist trafficking profiles may lead to differential brain uptake. For exendin-4 and exendin-asp3, which induce extensive GLP-1R internalisation, receptor-mediated delivery into the brain may be substantial. By contrast, exendin-phe1 results in slower and reduced overall levels of GLP-1R internalisation, and thus may exhibit reduced brain entry. Consequent reductions in access to and impact upon appetite-regulating centres may therefore explain why exendin-phe1 fails to show a bias-related enhancement in anorectic effects relative to the other two agonists. In addition to reduced overall levels of agonist-induced internalisation, exendin-phe1 exhibits a significantly lower acute rate of internalisation relative to exendin-4 and exendin-asp3. This phenomenon may be manifested physiologically as the delayed onset in exendin-phe1's anorectic effects (along with contributions from exendin-phe1's reduced acute potency as discussed in Section 3.2.3). Finally, the fact that brain-impermeant GLP-1RA albiglutide achieves less weight loss than smaller, brain-penetrant GLP-1RAs in head-to-head clinical trials [268] further substantiates a relationship between brain entry and anorectic efficacy.

Though a feasible hypothesis, an alternative scenario is that the agonists access CNS-located appetite regulatory centres equally, but here signalling responses are distinct from those induced in pancreatic β -cells. The notion that cellular context affects biased agonism is captured in the concept of “system bias” [342]. Differences in the level of receptor expression, the level of expression of different transducer and modulatory proteins (e.g. G-proteins, β -arrestins, GRKs), the strength of receptor coupling with downstream proteins, and the expression of related receptors may all alter the observed cellular response to the same ligand. The term “system bias” is largely used with reference to the *in vitro* screening of biased agonists. However, the concept it captures likely applies *in vivo* given that the relative expression of G-proteins and β -arrestins differs considerably between tissues [342], [392]. Thus, the systemic administration of an agonist may engender unique signalling responses in different tissues expressing the cognate receptor, with biologically relevant effects. This notion will hereafter be referred to as “tissue bias”.

Relating this to the biased GLP-1RAs tested here, if the neurons responsible for enacting the anorectic effects of GLP-1RAs express, for example, β -arrestins at lower levels than pancreatic β -cells, exendin-4, exendin-phe1 and exendin-asp3 may all recruit similarly low levels of β -arrestins in these neurons. A lack of significant differences in β -arrestin recruitment could therefore account for the loss of bias-related effects in agonist-induced anorexia. This represents a single theoretical tissue-specific difference, and equally plausible alternative explanations undoubtedly exist. In support of differences in signalling between β -cells and neurons is the large difference in the agonists’ “biological potency” for anti-hyperglycaemic and anorectic effects. Considerably lower doses were required for glycaemic effects, with the average ED_{50} being 30-fold lower than that for acute anorectic effects (Table 3.1). The agonists thus appear to be significantly more potent at β -cells *versus* neurons, suggestive of differential GLP-1R coupling to, and amplification of intracellular responses in the two cell types.

Both explanations – differences in brain entry, or tissue-specific signalling - are intriguing and will be explored in subsequent chapters.

4 Brain Entry of Biased GLP-1RAs

4.1 Introduction

In this chapter, brain and pancreas uptake of exendin-4, exendin-phe1 and exendin-asp3 are directly assessed to interrogate whether the observed divergence between their glucoregulatory and anorectic effects is due to differences in their access to central appetite regulatory centres.

4.1.1 Barriers between the blood and the brain

Proper brain connectivity and synaptic activity requires precise regulation of the neuronal milieu. As such, barriers exist separating the CNS from the blood, the latter of which can show large fluctuations in pH, ion, metabolite, and hormone concentrations based upon metabolic state. However, the effectiveness of these barriers and relative inaccessibility of the CNS to blood-borne substances poses a major challenge for drugs with CNS targets. As GLP-1RAs liraglutide and semaglutide are now being prescribed as treatments for obesity independent of T2DM, interest has increased in the entry and action of GLP-1RAs upon the brain, where their anorectic effects are believed to be mediated [163], [180].

The barriers, entry points and transport mechanisms into the CNS are summarised below, along with what is known or hypothesised about brain entry of GLP-1RAs. Discussion upon the latter topic is primarily concerned with pharmacological GLP-1 mimetics which persist in the circulation long enough to gain access to the CNS, unlike endogenous intestinal GLP-1, for which rapid degradation close to its source of production precludes significant delivery to the brain (see Section 1.6.2).

4.1.1.1 *The blood-brain barrier*

The primary structure achieving homeostatic control of the neuronal environment is the BBB: a neurovascular unit of highly-polarised endothelial cells (ECs) and associated pericytes, glia (astrocytes, microglia, oligodendroglia), neurons, and immune cells [390], [393], [394]. ECs form continuous, non-fenestrated capillaries with tight junctions between cells that prevent the paracellular entry of pathogens, blood cells and neurotoxic substances (e.g. plasminogen, thrombin, glutamate) [390], [395]. Whilst oxygen and carbon dioxide freely diffuse across the BBB, the ECs express multiple substrate-specific transport systems that control the influx of nutrients and metabolites, and efflux of metabolic waste products [390], [395]. Ion flux is also actively regulated at the BBB by specific channels and transporters to protect against electrolyte fluctuations that occur in the blood [390], [395]. These various protective and homeostatic functions of the BBB are all the more critical given the limited regenerative capacity of neurons, and the lack of adult neurogenesis [395].

4.1.1.2 *Circumventricular organs & the blood-CSF barrier (BCSFB)*

Although most of the CNS vasculature possess BBB properties, limited regions within the CNS - termed circumventricular organs (CVOs) - are supplied by highly-permeable fenestrated capillaries [394], [396]. CVOs lie adjacent to the third and fourth ventricles and, in mammals, are the area postrema (AP), median eminence (ME), choroid plexus (CHp), subfornical organ (SFO), organum vasculosum of the lamina terminalis (OVLT), subcommissural organ (SCO), pituitary gland, and pineal gland [396]. The reduced barrier properties of the supplying microvasculature allows these regions to detect circulating factors (sensory CVOs) and to secrete substances directly into the blood (secretory

CVOs) [394]. Sensory CVOs include the AP, OVLT and SFO, and secretory CVOs the pineal gland, pituitary gland and ME [395]. Of note, the CHp is the site of the blood-CSF barrier (BCSFB), a second blood-brain interface distinct from the BBB [395]. Unlike other CVOs that are single structures located adjacent to the third and fourth ventricles, a separate CHp exists in each of the four ventricles (third, fourth, and left and right lateral ventricles) [396]. Although the capillaries supplying the CHp are fenestrated, tight junctions between the ependymal cells of the CHp form the BCSFB and, as with the BBB, the passage of substances is actively regulated by substrate-specific transporters [397].

Akin to the CHp and against the notion of CVOs being unrestricted “brain windows”, a cellular barrier prevents the free movement of blood-borne substances from within CVOs to BBB-protected adjacent regions and ventricles [396], [398]. Supporting this, systemic injection of tracers results in their accumulation in CVOs, but a lack of spread further into the brain parenchyma [396]. This has been extensively demonstrated for the ME, with systemically administered tracers (trypan blue, Evans blue, ferritin, horseradish peroxidase) readily reaching the intercellular space of the ME but failing to spread to the neighbouring ARC [396], [399]. Thus, whilst the median eminence milieu and the perivascular space of the supplying capillaries are in open communication, tracing experiments suggest that this does not extend to the ARC (except under certain circumstances, see below). The cellular components of this barrier are specialised ependymal/glial cells called tanycytes. At the ME-third ventricle barrier, tight junctions between the β_2 subpopulation of tanycytes seal off the ME from the CSF, whilst between the ME and ARC, tight junctions between the long basal processes of β_1 tanycytes execute the barrier function [396], [399], [400] (Figure 4.1).

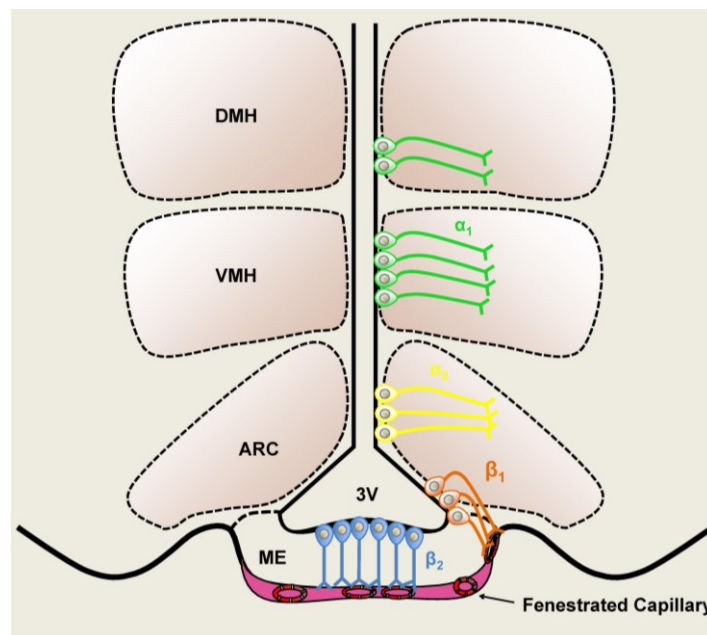


Figure 4.1. Schematic of tanycytic populations and their relative locations.

The relative locations of the α_1 , α_2 , β_1 , β_2 tanycyte populations that line the 3rd ventricle. α_1 tanycytes line the ventricular border of the VMH and part of the DMH and project their basal processes into these nuclei. α_2 tanycytes line the ventricular border of the ARC and project their processes into the ARC. β_1 tanycyte cell bodies are located in the dorsolateral ME and ventromedial ARC; their basal processes extend to the external regions of the ME where they contact fenestrated capillaries. Tight junctions between their basal processes form a barrier between the ME and ARC. β_2 tanycytes line the ventricular border of the ME and their basal processes contact fenestrated capillaries in the ME. Tight junctions between β_2 tanycyte cell bodies form a barrier between the ME and the 3rd ventricle. VMH: ventromedial nucleus of the hypothalamus. 3V: 3rd ventricle. ARC: arcuate nucleus of the hypothalamus. DMH: dorsomedial nucleus of the hypothalamus. ME: median eminence. VMH: ventromedial nucleus of the hypothalamus.

Similar observations are seen for the sensory CVOs, with peripherally injected tracer fluorescein isothiocyanate (FITC) or Evans blue dye readily entering the parenchyma of the AP, OVLT and SFO but being sequestered within these regions by a dense network of tight junction-connected tanycytes at the boundaries of the regions [398], [401]. Injection of Evans blue dye in the lateral ventricles fails to result in accumulation within the AP, SFO and OVLT, demonstrating the effectiveness of the tanycyte barrier at the ventricle-CVO interface [398]. Notably, the ARC is an exception to this, as the subpopulation of α_2 tanycytes that line the ARC-ventricle boundary do not possess tight junctions [396]. As such, intracerebroventricular (ICV) injection of Evans blue dye results in accumulation the ARC [402]. However, it does not spread to the adjacent ME or VMH, confirming the existence of an ARC-ME barrier [402]. Functionally, a lack of free passage from CVOs to adjacent nuclei and ventricles is important, as otherwise the brain would be subject to the unregulated entry of all compounds capable of entering CVOs [396]. Thus, at CVOs, the location and constituent cells of the barrier separating the blood and brain are different from the BBB proper. Rather than being at the vascular surface and composed of endothelial cells, a barrier composed of tanycytes resides at the ventricular and inter-regional boundaries.

It should be noted that – against the evidence above – immunohistochemical studies in the mouse and rat suggest that the ventromedial ARC (vmARC) is irrigated by fenestrated capillary loops of ME origin and therefore is able to directly sense blood-borne molecules [403], [404]. Whether these capillaries are permeable in all contexts is unclear, though there is functional evidence for their permeability specifically in the fasted state [405]. Fasting for 24 hours results in tanycytic rearrangements and a VEGF-dependent increase in the fenestration of ME capillary loops that extend into the vmARC, causing the vmARC to become permeable to blood-borne factors [405]. This is demonstrated by both the extended diffusion of intravascularly administered Evans blue dye into the ARC (restricted to the ME in the fed state), increases in ARC glucose concentration as measured by microdialysis in freely-moving animals, and increases in the sensitivity to endogenous circulating leptin as shown by a spontaneous reduction in food intake and increase in ARC-specific Leptin receptor (LepR) activity [405]. This fasting-specific increase in vmARC permeability is corroborated by a significant increase in the presence of fluorescently-tagged ghrelin detected in the vmARC after 24 hours of fasting [406]. Thus, a subset of capillaries extending in the vmARC exhibit nutritional status-dependent permeability changes, thereby facilitating direct sensing of circulating hormones by the vmARC in the fasted state.

4.1.2 Transport into the brain

The presence of tight junctions between BBB endothelial cells, and between ependymal cells at the BCSFB and CVOs prevents the paracellular diffusion of substances, meaning that transport into the brain must occur transcellularly. Multiple mechanisms exist, with that being engaged being determined by the size and other physicochemical properties of the substance in question [395]. As the vast majority of research has been focussed upon the BBB, discussion below will pertain to this structure rather than the BCSFB or CVOs unless specifically stated. Transport mechanisms across the BCSFB and at CVOs are less well understood, though it is asserted that similar systems exist to the BBB [407]. Historical focus upon the BBB has been due to the widely-held belief that it constitutes the largest interface for blood-brain exchange [395], though morphometry analyses suggest that the choroid plexus boasts a surface area of comparable size and importance for exchange [396].

Of note, the neurovascular unit of the BBB is a dynamic structure that senses the microenvironment and responds to the needs of the CNS [393]. Crosstalk between ECs, astrocytes and neurons can lead to changes in BBB permeability [393].

4.1.2.1.1 Passive Diffusion

The major physicochemical properties determining whether substances can diffuse across the BBB are molecular mass, lipophilicity/lipid solubility, hydrogen bonding, and electrostatic charge [395]. Small, lipophilic molecules with a molecular weight of less than 400-450 Da, fewer than 6-8 hydrogen bonds and a polar surface area of less than 80 Å² can freely cross the BBB [390], [395]. This includes blood gases oxygen and carbon dioxide, and their supply and removal, respectively, is dependent upon cerebral blood flow [395]. Passive diffusion represents a non-saturable mechanism for brain uptake.

4.1.2.1.2 Carrier-mediated transport

Polar nutrients such as glucose and amino acids that are essential for metabolism are unable to diffuse across the BBB and rely upon the presence of carrier-mediated transport (CMT) systems to enter the CNS. CMT is enacted by membrane transport proteins of the ubiquitously-expressed solute carrier (SLC) family, and involves the movement of substances down their concentration gradient (i.e. facilitated diffusion) [390], [394]. Endothelial cells of the BBB express many SLC proteins, facilitating the transcellular transport of carbohydrates, amino acids, hormones, fatty acids, nucleotides, organic ions, choline, amines, vitamins, and nucleotides [390], [395]. Expression of SLC proteins in the luminal and/or abluminal membranes determines the directionality of transport, and is substrate-specific [395].

4.1.2.1.3 Transcytosis

Transcytosis via endocytic mechanisms represents the main route that large molecular weight substances such as proteins can enter the CNS, and takes the form of receptor-mediated transcytosis (RMT), adsorptive-mediated transcytosis (AMT), or pinocytosis [395]. In RMT, ligand binding to a specific receptor induces endocytosis of the receptor-ligand complex into vesicles, before the vesicle is routed across the cytoplasm and exocytosed at the opposite pole of the cell. By contrast, AMT is receptor-independent, and relies upon excess positive charge on the surface of the molecule to allow it to interact with negatively-charged cell surface binding sites and induce endocytosis. Pinocytosis, a non-specific means of endocytosis, may also be employed for smaller peptides [395]. Endocytosis by BBB ECs involves both caveolin-based and clathrin-coated vesicle trafficking [408], [409] .

Compared with peripheral ECs, BBB ECs undergo significantly lower rates of endocytosis, which may limit transcytosis as a means of transport into the brain [394]. Additionally, the mechanisms by which vesicles are routed away from the endocytic pathway (and subsequent lysosomal degradation, or recycling to the entry membrane) and transported to the opposite cellular pole are poorly understood. Recent work suggests intracellular tubules facilitate transcytosis in RMT, and that binding affinity/avidity between the receptor and molecule being transported partly determines whether lysosomal degradation or successful transcytosis occurs [410]. Despite the dearth of mechanistic understanding, transcytosis is an established mechanism for brain entry at the BBB for a number of

molecules, as detailed in Table 4.1. Supporting its relevance as a means of brain entry, endogenously-occurring RMT is currently being exploited to enhance the delivery of therapeutics into the brain [411]. Successful preclinical studies include the delivery of an anti-A β -antibody via the transferrin receptor (TfR) [412], the TfR-dependent delivery of anti-apoptotic peptides in nanoparticles coated in anti-TfR antibodies [413], and the delivery of anti-cancer drug paclitaxel via conjugation to LRP-1-binding peptide “angiopep-2” [414].

Receptor	Abbreviation	Ligand	Direction of Transport
Vassopressin V1 receptor	V1	Arginine-vasopressin	Bidirectional
Transferrin receptor	TfR	Transferrin	Blood to brain
Lactoferrin receptor	LfR	Lactoferrin	Blood to CSF
Leptin receptor	LepR	Leptin	Blood to brain
Insulin receptor	IR	Insulin	Blood to brain
Low density lipoprotein (LDL)-receptor-related protein 1	LRP1	Apolipoproteins (e.g. amyloid- β , lactoferrin, ApoE)	Brain to blood
LDL-receptor-related protein 2 (megalin)	LRP2		
Receptor for advanced glycosylation end-products	RAGE	Glycosylated proteins	Blood to brain
Fc-gamma receptor	Fc γ R	Immunoglobulin G	Blood to brain
Tumour necrosis factor receptor	TNFR	TNF α	Blood to brain
Epidermal growth factor receptor	EGFR	EGF	Blood to brain

Table 4.1 Examples of transcytosis systems in endothelial cells of the BBB.

From [390], [395].

Although the BBB ECs have been shown to have low endocytic capacity, electron microscopy studies reveal a high density of both clathrin-coated and non-clathrin-coated vesicles in choroidal ependymal cells at both the apical and basolateral membranes [415]. Evidence for RMT at the BCSFB is a less robust than at the BBB, though the data suggest the existence of systems involving the TfR, LRP1, LRP2 and LRP8, as well as BCSFB-specific systems involving the folate receptor α (FR α) [415]. Of note, multi-ligand receptor LRP2 has been shown to transport insulin growth factor-1 [416] and leptin [417] across the BCSFB.

More recently, the uptake of blood-borne leptin has been shown to occur through tanycyte-mediated transcytosis [391]. Peripherally administered fluorescently-labelled leptin enters the ME through fenestrated capillaries and is taken up by ME tanycytes in a LepR-dependent manner, before being transported up tanycytic basal processes and released into the CSF of the third ventricle [391]. Once in the CSF, it accesses the adjacent ARC (given the differences in tanycytic barrier properties – see above), as well as more distant, CSF-contacting sites the hippocampus, cerebral cortex, and medial nucleus of the amygdala [391]. Detection of fluorescently-tagged ghrelin exclusively in ME tanycytes and the ARC shortly after intravenous administration suggests that tanycytes mediate CNS entry of

ghrelin in a similar fashion to leptin [418], [419]. These studies suggest that tanycytes may act as a general conduit for circulating metabolic hormones.

4.1.2.1.4 ABC transporter efflux

BBB endothelial cells express multiple ATP-binding cassette (ABC) proteins on the luminal (blood-facing) membrane that actively efflux substances from the CNS. In the BBB, the main ABC transporters are P-glycoprotein (Pgp – luminal expression), the multidrug resistance proteins (MRPs – luminal and abluminal expression), and breast cancer resistance protein (BCRP – luminal expression) [420]. The BCSFB also expresses efflux pumps, though a partially different set compared to the BBB [415]. Importantly, these transporters show a striking lack of substrate selectivity, and their presence underlies the observation that the CNS penetrance of many substances is lower than would be predicted based on their physicochemical properties [395], [420]. ABC transporters play a critical neuroprotective function by removing neurotoxic endogenous or xenobiotic molecules. However, their presence also accounts for CNS pharmacoresistance, and the challenge of increasing drug bioavailability in the CNS has proven to be non-trivial.

4.1.2.2 CNS entry of GLP-1RAs?

A critical determinant of drug efficacy is achieving adequate bioavailability at the site of action. The presence of the BBB has proved a major impediment to drugs with CNS targets, and numerous modifications have been applied to try and increase brain penetrance. These include pegylation, addition of fatty acids, glycosylation, conjugation to receptor-targeting peptides or antibodies (to enhance RMT), and cationisation (to increase AMT) [421]. However, the overall effect of a modification is not easily predicted. For example, increasing the lipid solubility of a substance would be predicted to increase penetrance given more favourable physicochemical properties for diffusion. However, a decrease in CNS entry may be observed due to increased uptake by peripheral tissues, an increased propensity to be a substrate for efflux pumps, and trapping in the lipid membranes of BBB ECs [421]. Likewise, binding to plasma proteins has been attributed to poor brain penetrance (e.g. of indomethacin [422]) due to decreasing the free fraction of substance capable of transport across the BBB. However, protein binding can increase circulating half-life and thereby increase the time and concentration of drug exposure to the BBB [421]. Thus, the relationship between drug properties and CNS entry is complex, and still incompletely understood.

It is widely accepted that therapeutic GLP-1RAs successfully cross the BBB, and that their anorectic effects are – at least in part – exerted by direct action upon central GLP-1Rs. In contrast, it is believed that GLP-1 produced by intestinal L-cells promotes satiety primarily through action on GLP-1R-expressing vagal afferent neurons in the vicinity of its site of production, as rapid enzymatic degradation severely limits its potential endocrine effects (see Section 1.6.2). Early studies demonstrated the ability of a DPP-4-resistant form of GLP-1 and of exendin-4 to cross the BBB in a rapid manner reminiscent of diffusion [194], [423]. Moreover, the neuroprotective and neurotrophic properties of GLP-1 and GLP-1RAs have been long-recognised, and their success in preclinical studies and clinical trials for neurodegenerative diseases is believed to be related, at least in part, to their ability to cross the BBB [424]. Notably, unlike GLP-1RAs, other neuroprotective growth factors (e.g. nerve growth factor, brain-derived neurotrophic factor) do not cross the BBB, and thus do not confer neuroprotective effects after peripheral administration [424].

As highlighted above, a number of studies point to the importance of central over peripheral GLP-1Rs in the anorectic effects of long-lasting GLP-1RAs, with the anorectic and weight-lowering effects of liraglutide being specifically abolished by central, but not peripheral, knockout of neuronal GLP-1Rs [180], or by ablation of the GLP-1R-expressing arcuate nucleus, but not by subdiaphragmatic vagal deafferentation [179]. It should be noted however that Kanoski et al. found both central and peripheral GLP-1Rs to contribute to the anorectic effects of exendin-4 and liraglutide [196]. The role of central GLP-1Rs in hypophagia induced by pharmacological agents may explain why albumin-conjugated GLP-1RA albiglutide, the brain entry of which may be impeded by its molecular weight, results in poorer weight loss than other, BBB-penetrant GLP-1RAs [268], [425], [426]. Corroborating this, a recent study demonstrated that deletion of the GLP-1R from vagal neurons blunted the anorectic effects of albiglutide to a larger degree than deletion from CNS neurons; the reverse was true for exendin-4, with its anorectic effects still being significant at 24 hours after vagal GLP-1R deletion [163]. In further support for direct action on central GLP-1Rs, fluorescently-tagged liraglutide shows accumulation in both CVOs and BBB-protected regions after peripheral administration [179], [427], and these regions correlate strongly with those expressing the GLP-1R and showing liraglutide-induced neuronal activation [427]. Contrasting with these observations, a study in T2DM patients found that treatment-induced weight loss tended to correlate with plasma rather than CSF levels of liraglutide, suggesting that CNS entry is not required for liraglutide's weight-lowering effect [428].

This last study aside, the general consensus is that long-lasting GLP-1RAs readily cross the BBB and act directly upon central GLP-1RAs to exert their anorectic (and neuroprotective) effects. However, the mechanism of transport – passive diffusion, facilitated diffusion, or transcytosis (RMT or AMT) – is yet to be established. Kastin and Akerstrom report exendin-4 entry into the brain to be independent of CVOs and involving direct penetration of the BBB, though note that it displays unusual entry kinetics, with results suggesting passive entry except at high doses, where weak self-inhibition is observed [423]. The authors conclude that a saturable transport mechanism may be employed by exendin-4 only at high concentrations; otherwise the physicochemical properties of exendin-4 – with high lipophilicity and low hydrogen bonding – are permissive for passive diffusion [423]. Exendin-4's entry kinetics are markedly different to leptin and insulin which show strong self-inhibition at much lower doses, consistent with saturable transport mechanism [429], [430]. In keeping with these early kinetic studies, RMT-mediated mechanisms for both insulin and leptin have been demonstrated [395].

Despite suggestion that exendin-4 may enter the CNS in a non-saturable manner (except at high concentrations), its molecular weight (4187 Da) is notably higher than the optimal molecular weight of <500 Da for passive brain entry [390], [431]. However, non-saturable brain entry of similarly sized or even larger endogenous, satiety-related peptides such as NPY [432], nesfatin [433] and FGF21 [434] has been demonstrated [435]. Thus, and as aforementioned, though generalisations are made about molecular properties favourable for brain entry, they do not rigidly apply.

More recently, suggestions have been made that brain entry of GLP-1RAs occurs in a receptor-dependent manner. This is largely from the observation that accumulation of fluorescently-tagged liraglutide in the brain is not observed in *Glp1r*^{-/-} mice [179]. The authors of this paper suggest that tanycytes may mediate brain uptake of liraglutide in a GLP-1R-dependent endocytic and transcytic manner, similar to leptin and ghrelin. However, it should be noted that the lack of detection in the absence of the GLP-1R may not evidence its necessity for brain uptake, as accumulation of liraglutide in brain regions (and therefore fluorescence detection) is likely dependent upon receptor binding

alone. In summary, though it is generally accepted that pharmacological GLP-1RAs enter the brain and act upon central GLP-1RAs, the precise mechanisms by which brain entry is achieved and regulated are poorly understood.

4.1.3 Tissue clearing

The experiments featured in this chapter utilise tissue clearing – the process of making large, fixed biological samples transparent - to examine signal from fluorescently-tagged forms of the agonists in whole mouse brains and pancreata. The fact that biological tissue is opaque has previously restricted histological investigation to the labour-intensive process of slicing samples into thin sections (around 10 to 30 μm). With this approach, three-dimensional tissue structure is lost, and whilst it can be reconstructed from a set of serial sections, the process of doing so is both technically challenging and may yield inaccurate results [436]. Confocal and two-photon microscopy partly overcome this by isolating information from a single optical plane in a thick tissue slice (i.e. minimising contamination from out-of-focus light arising from other planes in the sample). The tissue slice can therefore be “optically sectioned” into a stack of images that are then reconstructed into a 3D volume. However, this approach is still limited to sections that are hundreds of microns thick. By contrast, tissue clearing facilitates the imaging of whole organs, and even entire organisms [437].

Biological tissue is composed of a diverse array of cellular and extracellular components arranged in a non-homogenous manner. Each of these components has a different refractive index (the speed of light in the medium of interest relative to that in a vacuum), causing light propagating through the tissue to be scattered and thus rendering the tissue opaque or translucent. The process of tissue clearing homogenises the refractive index of the sample to the surrounding medium by removing or modifying tissue components, thereby preventing light scattering [436]. A large number of tissue clearing methods have been developed in the past 7 years, with four main categories based upon a shared chemical approach: organic solvent-based clearing, aqueous solvent-based clearing, hyperhydration, and hydrogel-embedding [436]. Here, an organic solvent-based method was used due to rapidity and quality of the clearing achieved. This category of clearing involves tissue dehydration and mild delipidation in one set of solutions, followed by extensive delipidation and refractive index matching with the remaining, dense proteinaceous structure in a second solution. Delipidation removes cell plasma membranes: one of the main sites of tissue inhomogeneity and therefore contributors to light scattering [436]. The result is a remarkably clear structure suitable for visualisation by optical projection tomography, confocal microscopy, two-photon microscopy, or light sheet imaging, with the imaging method of choice being based upon the desired resolution and physical size of the sample [436].

In summary, tissue clearing is a powerful approach to obtain valuable, three dimensional data about whole tissues and organisms by overcoming the photonic limitations posed by opaque tissue. The rate at which refined, or wholly new clearing approaches have been developed over the past 5 years is testament to its applicability to a broad range of biological questions.

4.2 Aims

1. To assess whether differences exist in the extent of entry or distribution of the biased GLP-1RAs in the brain after peripheral administration.
2. To assess whether brain entry of GLP-1RAs is associated with agonist-specific differences in GLP-1R endocytosis.

4.3 Results

4.3.1 The design of sCy7-tagged GLP-1RAs

The experiments in this chapter utilise fluorescently-tagged forms of exendin-4, exendin-phe1 and exendin-asp3 to compare brain entry of biased GLP-1RAs after whole brain tissue clearing. The ability to visualise fluorescent markers in tissue is often hindered by tissue autofluorescence arising from both endogenous tissue elements (e.g. collagen, lipofuscin), as well as tissue processing techniques (e.g. formalin fixation) [438]. Imaging of entire tissues in three dimensions augments this issue: autofluorescence will summate through millimetres of tissue as opposed to microns in tissue slices. As tissue autofluorescence is most severe in the shorter fluorescence wavelengths (blue, green and red fluorescence), near-infrared fluorophore sulfo-cyanine 7 azide (sCy7; peak excitation 750 nm, peak emission 773 nm) was chosen to be conjugated to the peptides. The water-soluble “sulfo” form was used as the physicochemical properties of lipophilic “non-sulfo” Cy7 have been shown to affect ligand pharmacokinetics via changes to plasma protein binding [439]. To enable conjugation, peptides were synthesised with non-proteinogenic amino acid L-propargylglycine (“pra”) at position 12 (replacing endogenous lysine). Pra bears an alkyne side chain, allowing conjugation to the fluorophore’s azide group via copper-catalysed alkyne-azide cycloaddition (CuAAC) “click” chemistry [440], [441]. Position 12 was chosen for fluorophore conjugation given previous reports of conjugation at this location having minor to no effect upon exendin-4 binding affinity and potency at the GLP-1R [323], [441]–[443]. Synthesis and purification of the sCy7-conjugated GLP-1RAs and all other fluorescently-tagged peptides in this chapter was kindly conducted by Dr Masha Shchepinova, Imperial College London.

4.3.2 *In vitro* signalling of pra12-modified and sCy7-tagged GLP-1RAs

As demonstrated by the biased agonists tested in this project, single amino acid substitutions may dramatically alter agonist properties. Moreover, the covalent attachment of fluorophores and other functional groups (e.g. fatty acids) can influence receptor pharmacology by sterically or electrostatically hindering normal receptor binding. cAMP accumulation responses and agonist-induced internalisation were therefore tested for both the pra12-modified agonists and their sCy7-tagged derivatives in comparison to their unmodified forms, to establish whether the click chemistry-compatible pra12 change or the fluorophore itself exerted deleterious effects on agonist performance.

Substitution of lysine to propargylglycine at position 12 had minimal effects upon agonist potency and efficacy for cAMP accumulation (Figure 4.2, Table 4.2). Agonist-induced internalisation was also similar for pra12-modified agonists relative to their unmodified counterparts (Figure 4.3). Exendin-4-pra12 and exendin-asp3-pra12 showed extensive internalisation whilst exendin-phe1-pra12 showed minimal internalisation. Therefore, the pra12 substitution is well tolerated in all three base peptides, in keeping with prior results with exendin-4 [441], [442].

By contrast, conjugation of sCy7 significantly affected cAMP accumulation signalling potency and in an agonist-dependent manner: exendin-4-pra12-sCy7 showed a 17.3-fold decrease in potency, exendin-phe1-pra12-sCy7 a 50.2-fold decrease, and exendin-asp3-pra12-sCy7 a 5.95-fold decrease (Figure 4.4, Table 4.3). Exendin-phe1-pra12-sCy7 also exhibited decreased efficacy relative to exendin-phe1. Thus, as anticipated, fluorophore conjugation resulted in decreases in agonist potency,

as well as efficacy in certain cases, with exendin-phe1 being most significantly affected. Agonist-induced internalisation was similar to unmodified compounds for exendin-phe1-pra12-sCy7 and exendin-asp3-pra12-sCy7, although exendin-4-pra12-sCy7 showed reduced internalisation more akin to exendin-phe1-based agonists (Figure 4.5). The impact of these signalling changes on *in vivo* responses will need to be determined, especially given that the agonists were differentially affected in both assays.

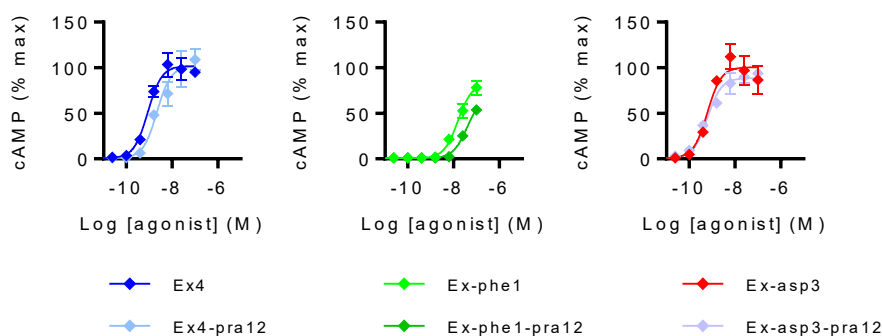


Figure 4.2. cAMP accumulation responses to pra12-modified GLP-1RAs.

cAMP response to unmodified and pra12-modified exendin-4, exendin-phe1 and exendin-asp3 in HEK293-SNAP-GLP-1R cells, 30 min incubation. Data are expressed as a percentage of the maximal response in the assay with four-parameter logistic fit of data.

Peptide	pEC ₅₀	ΔlogEC ₅₀	EC ₅₀ Ratio	E _{max} (%)	Ratio
Ex4	9.08 ± 0.05			102 ± 6.37	
Ex-phe1	7.61 ± 0.10			85.6 ± 8.37	
Ex-asp3	9.21 ± 0.03			102 ± 3.00	
Ex4-pra12	8.64 ± 0.12 *	0.438 ± 0.35	2.74	112 ± 9.51	1.10
Ex-phe1-pra12	7.09 ± 0.02	0.523 ± 0.05	3.34	75.6 ± 12.7	0.88
Ex-asp3-pra12	9.05 ± 0.05	0.165 ± 0.02	1.46	88.4 ± 4.56	0.86

Table 4.2. Potency and efficacy of unmodified and pra12-modified GLP-1RAs for cAMP accumulation.

Values derived from data in Figure 4.2. cAMP accumulation responses to pra12-modified GLP-1RAs. “ΔlogEC₅₀” is the difference in logEC₅₀ values between unmodified and pra12-modified counterparts. “EC₅₀ Ratio” describes the ratio between the EC₅₀ values for unmodified and pra12-modified counterparts. “Ratio” denotes E_{max} value relative to that of the equivalent unmodified compound. E_{max} values are normalised to global maximum in individual experiments. Significance reported in comparison to unmodified peptide e.g. ex4 vs. ex4-pra12. One-way randomised block ANOVA with Tukey’s test. *p<0.05. Data reported as mean ± SEM from 4 independent experiments.

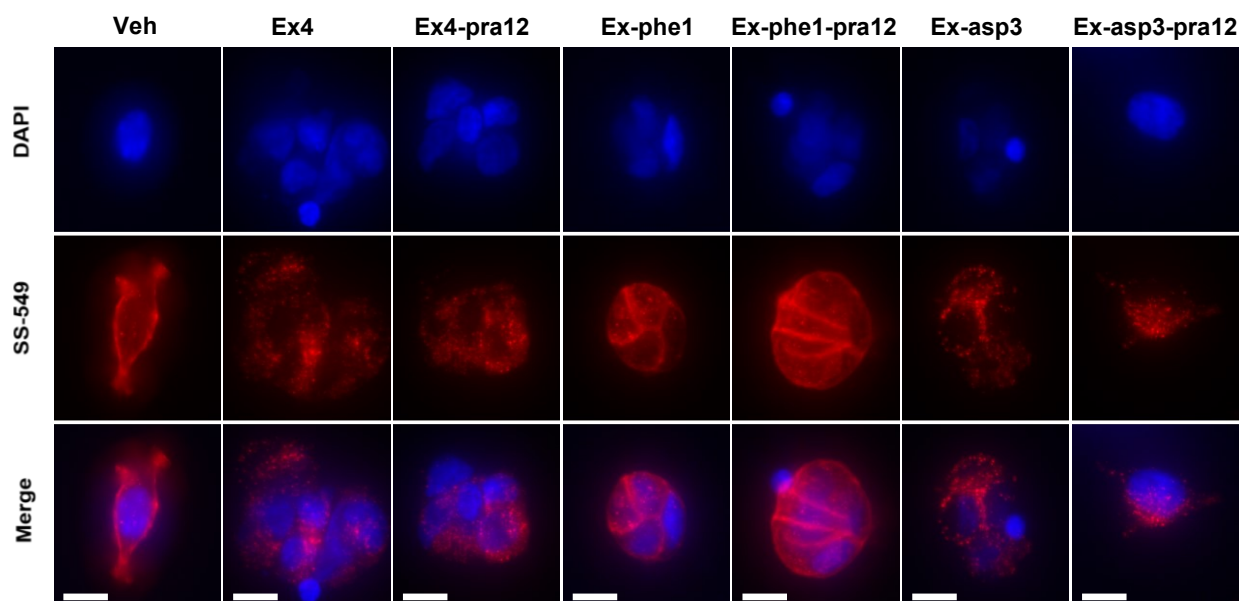


Figure 4.3. GLP-1R internalisation in response to pra12-modified GLP-1RAs.

Representative images indicating internalisation of SNAP-GLP-1R labelled with SNAP-Surface 549 ("SS-549"; red) after 30 minutes of agonist stimulation at 100 nM in HEK293-SNAP-GLP-1R cells. n=3 experiments, scale bar denotes 8 μ m.

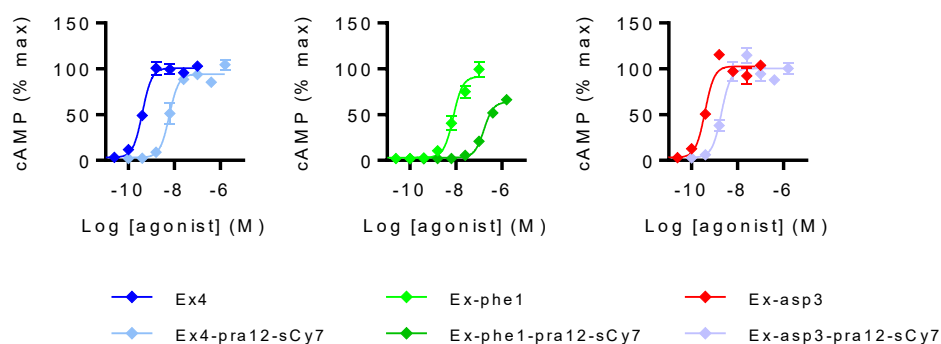


Figure 4.4. cAMP accumulation responses to sCy7-tagged GLP-1RAs.

cAMP response to unmodified and sCy7-tagged exendin-4, exendin-phe1 and exendin-asp3 in HEK293-SNAP-GLP-1R cells, 30 min incubation. Data are expressed as a percentage of the maximal response in the assay with four-parameter logistic fit of data.

Peptide	pEC ₅₀	Δ logEC ₅₀	EC ₅₀ Ratio	E _{max} (%)	Ratio
Ex4	9.41 $\pm$ 0.05			102 $\pm$ 2.44	
Ex-phe1	8.03 $\pm$ 0.11			94.2 $\pm$ 7.81	
Ex-asp3	9.47 $\pm$ 0.11			104 $\pm$ 2.62	
Ex4-pra12-sCy7	8.17 $\pm$ 0.12 *	1.34 $\pm$ 0.06	17.3	95.6 $\pm$ 2.48	0.94
Ex-phe1-pra12-sCy7	6.33 $\pm$ 0.07 ***	1.70 $\pm$ 0.03	50.2	66.4 $\pm$ 4.58	0.71
Ex-asp3-pra12-sCy7	8.70 $\pm$ 0.07 **	0.77 $\pm$ 0.03	5.95	102 $\pm$ 1.35	0.98

Table 4.3. Potency and efficacy of unmodified and sCy7-tagged GLP-1RAs for cAMP accumulation.

Values derived from data in Figure 4.4. “ $\Delta\log EC_{50}$ ” is the difference in $\log EC_{50}$ values between unmodified and sCy7-modified counterparts. “ EC_{50} Ratio” describes the ratio between the EC_{50} values for unmodified and sCy7-modified counterparts. “Ratio” denotes E_{max} value relative to that of the equivalent unmodified compound. E_{max} values are normalised to global maximum in individual experiments. Significance reported in comparison to unmodified peptide e.g. ex4 vs. ex4-pra12-sCy7. One-way randomised block ANOVA with Tukey’s test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data reported as mean $\pm$ SEM from 4 independent experiments.

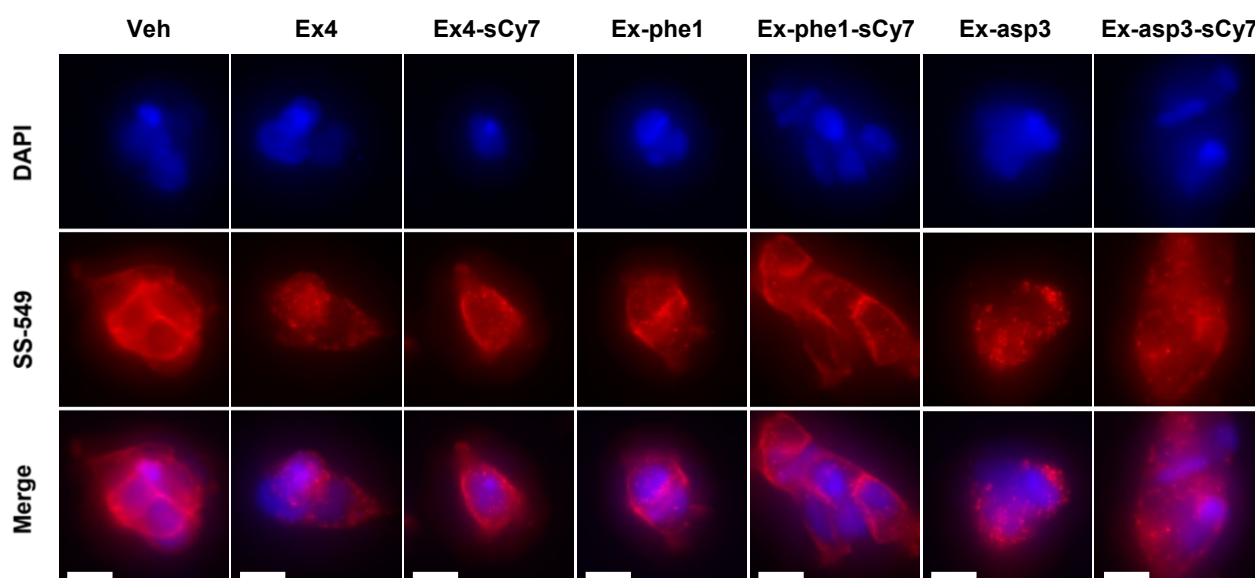


Figure 4.5. GLP-1R internalisation in response to sCy7-tagged GLP-1RAs.

Representative images indicating internalisation of SNAP-GLP-1R labelled with SNAP-Surface 549 (“SS-549”; red) after 30 minutes of agonist stimulation at 100 nM in HEK293-SNAP-GLP-1R cells. $n = 3$ experiments, scale bar denotes 8 μm .

4.3.3 Glycaemic responses to pra12-modified and sCy7-tagged GLP-1RAs

To ascertain whether bias-related physiological responses were preserved after agonist modification, 6 hour delayed IPGTTs were conducted with the pra12-modified and sCy7-tagged agonists. Glycaemic responses to the pra12-modified agonists were similar to unmodified biased agonists at 3 nmol/kg, with exendin-phe1-pra12 being most, and exendin-asp3-pra12 being least anti-hyperglycaemic. (Figure 4.6 A). At a supramaximal dose (100 nmol/kg), exendin-phe1-pra12 ceased to outperform exendin-4-pra12, presumably as this high agonist dose was sufficient to fully activate any residual GLP-1Rs despite more extensive desensitisation with exendin-4-pra12. A similar trend was observed in Chapter 3 (Figure 3.4) and Chapter 6 (Figure 6.10). Exendin-pra12-asp3 remained the least effective even at the higher dose, in keeping with the more pronounced desensitisation seen with this ligand. Overall, and consistent with the minimal changes in signalling responses *in vitro*, a lysine to propargylglycine substitution at position 12 did not appreciably affect agonist properties *in vivo*.

However, glycaemic responses after administration of sCy7-tagged agonists indicated that the loss of potency observed *in vitro* was manifested *in vivo*. At 3 nmol/kg, G-protein-biased exendin-phe1-pra12-sCy7 failed to mitigate the glucose excursion whilst exendin-4-pra12-sCy7 and β -arrestin-

biased exendin-asp3-pra12-sCy7 had similar and significant anti-hyperglycaemic effects (Figure 4.6 B). Exendin-phe1-pra12-sCy7's lack of effect was likely due to its significantly reduced signalling potency (Table 4.3), as increasing its dose to 10 and then 100 nmol/kg correspondingly increased its anti-hyperglycaemic effects. Exendin-4-pra12-sCy7's apparently improved effects relative to exendin-4-pra12 at 3 nmol/kg (albeit without a direct head-to-head comparison) may be related to the reduced level of agonist-induced internalisation observed with the former (Figure 4.5), i.e. exendin-4-pra12-sCy7 exhibits some of the ostensibly beneficial characteristics of "standard" G-protein biased ligands such as exendin-phe1. Interestingly however, exendin-asp3-pra12-sCy7 was the most effective ligand at all doses tested, contrasting with the typical effects seen with non-modified exendin-asp3 and also with exendin-asp3-pra12 (Figure 4.2). The reason for this discrepancy is not clear. It is possible that subtle reductions in GLP-1R internalisation exist with this ligand but were not detected due to assay insensitivity. A further possibility is that the fluorophore may prolong the ligand pharmacokinetics as previously described for non-sulfoCy7 [439], meaning that at the 6-hour time point, sufficient agonist remains to fully activate residual GLP-1Rs despite marked desensitisation.

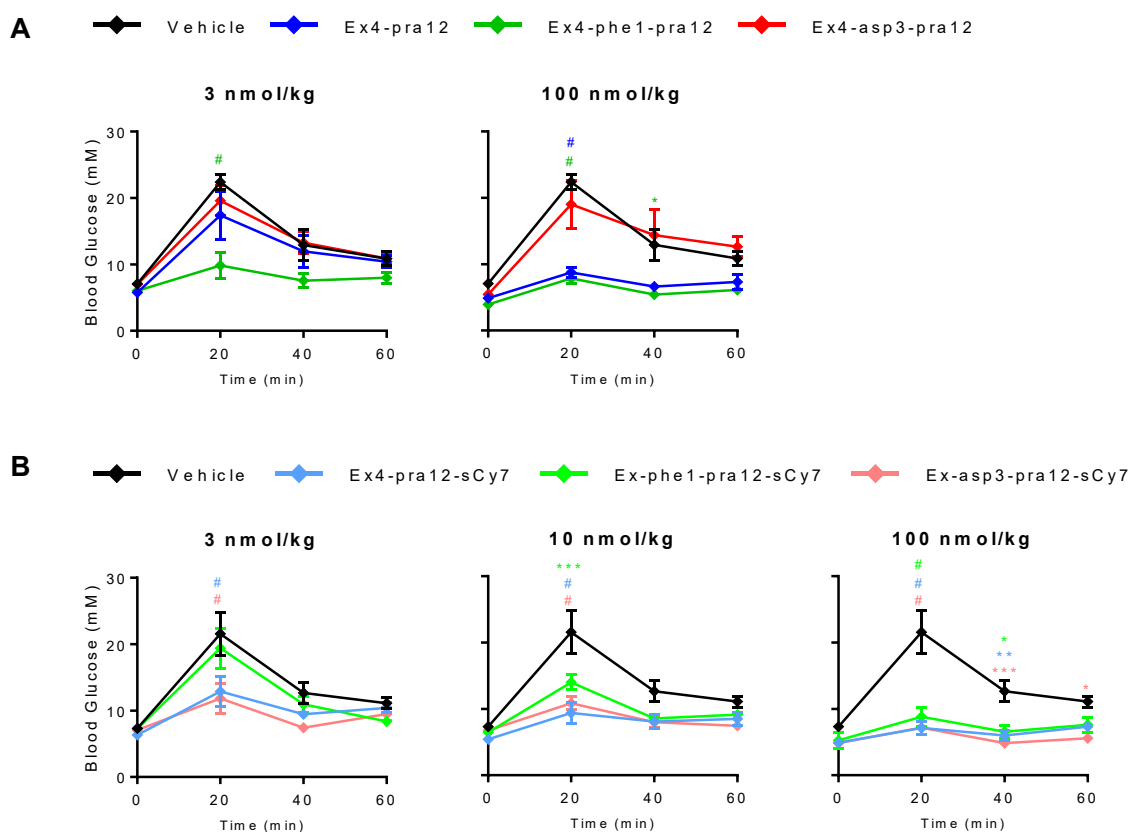


Figure 4.6. Glycaemic responses after administration of pra12-modified and sCy7-tagged GLP-1RAs.

(A) Blood glucose during IPGTT (2 g/kg glucose) in lean, male C57Bl/6 mice with glucose challenge 6 hours after intraperitoneal injection of pra12-modified agonists at 3 or 100 nmol/kg, n=5-6. **(B)** As for (A) but with sCy7-tagged agonists at 3, 10 or 100 nmol/kg, n=4. Two-way ANOVA with Tukey's test, with significance vs. saline shown. *p<0.05, **p<0.01, ***p<0.001, #p<0.0001. Data represented as mean $\pm$ SEM.

The loss of the expected rank order of anti-hyperglycaemic efficacy after fluorophore conjugation needs to be borne in mind for studies assessing brain entry using the sCy7-tagged agonists. As the bias-related physiology of the unmodified agonists is strongly correlated with their trafficking profiles, it

is likely that the changes in physiological responses to the sCy7-tagged agonists reflect changes in receptor trafficking relative to the unmodified agonists. Indeed, agonist-induced GLP-1R internalisation was modified at least for exendin-4-pra12-sCy7 (Figure 4.5), and more subtle changes to the properties of the other two sCy7-tagged agonists may have been missed due to the lack of sensitivity and non-quantitative nature of the assessment. Moreover, other aspects of trafficking (e.g. receptor recycling) were not studied and may also be modified. Thus, if brain entry of exendin-4-based GLP-1RAs involves receptor endocytosis and transcytosis, it may be the case that the movement of sCy7-tagged agonists across the BBB does not fully reflect that of the unmodified agonists. Therefore, any conclusions drawn about the relative brain entry of the sCy7-tagged agonists cannot be definitively linked to food intake responses to the unmodified agonists.

4.3.4 Brain entry of sCy7-tagged GLP-1RAs

Bearing the caveat above in mind, brain entry of sCy7-tagged agonists was compared by administering sCy7-tagged agonist at 100 nmol/kg to mice by intraperitoneal injection before transcardial perfusion with paraformaldehyde one or six hours later. The dose was selected after discussion with Dr Casper Bo Gravesen Salinas, Novo Nordisk [427]. Brains were collected, optically cleared using 3DISCO [382], and then imaged using optical projection tomography to assess the agonist apparent concentration and distribution in the brain. A representative image of a cleared mouse brain is shown in Figure 4.7, and representative images for each agonist at both time points are shown in Figure 4.9.

Abundant signal from all sCy7-tagged agonists was detected in CVOs including the median eminence (ME), area postrema (AP), vascular organ of the lamina terminalis (OVLT) and the subfornical organ (SFO). Moreover, careful inspection showed that signal was also seen in the arcuate nucleus (ARC) of the hypothalamus and the nucleus of the solitary tract (NTS) of the brain stem, indicating that agonist had spread beyond the presumed barrier which separates the CVOs from other brain regions. This is best shown on the maximum intensity projections in the dorsoventral plane in Figure 4.9; for example, the ring-like structure of the ARC can be clearly identified in continuity with median eminence signal seen in the midline. Total agonist uptake was quantified by summing the intensity value of all voxels in each region above a threshold intensity of 5000 units (to prevent inclusion of tissue autofluorescence and derived empirically by measuring signal in equivalent brain regions from vehicle-treated mice devoid of specific agonist signal). These values reflect both the intensity of agonist signal, as well as the total volume occupied by the agonist. Due to a lack of clear border between the ARC and ME, and the AP and NTS, signal in these pairs of regions were quantified together, being reported as the hypothalamus (HYP – ARC and ME) and dorsal vagal complex (DVC – AP and NTS). Quantification data are reported in Figure 4.8.

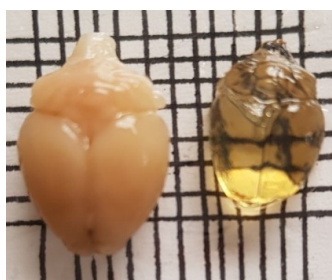


Figure 4.7. Tissue clearing of whole mouse brains.

A representative image of an unclarified (left) and 3DISCO-cleared (right) mouse brain.

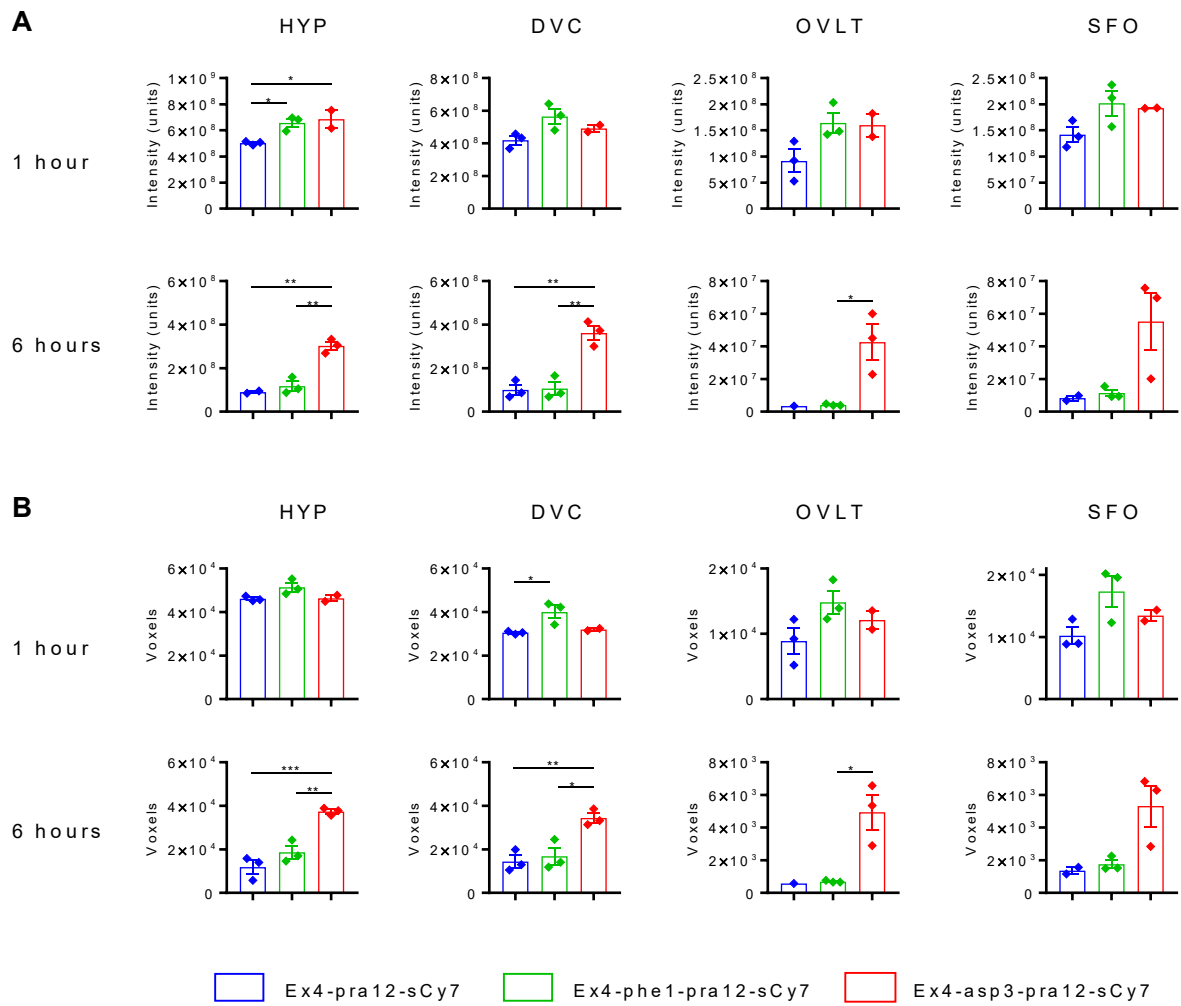


Figure 4.8. Quantification of sCy7-tagged agonist levels and volume in individual brain regions.

(A) Total fluorescence signal from the sCy7-tagged GLP-1RAs in the hypothalamus (ARC and ME), DVC (AP and NTS), OVLT and SFO at 1 and 6 hours post administration at 100 nmol/kg (n=2-3). Quantification was conducted using Volocity 6.3.0 by summing intensity values for all voxels within the region with an intensity value >5000 units. **(B)** As in (A) but reporting total volume of fluorescent signal. One-way ANOVA with Tukey's test. *p<0.05, **p<0.01, ***p<0.001. Data represented as mean $\pm$ SEM.

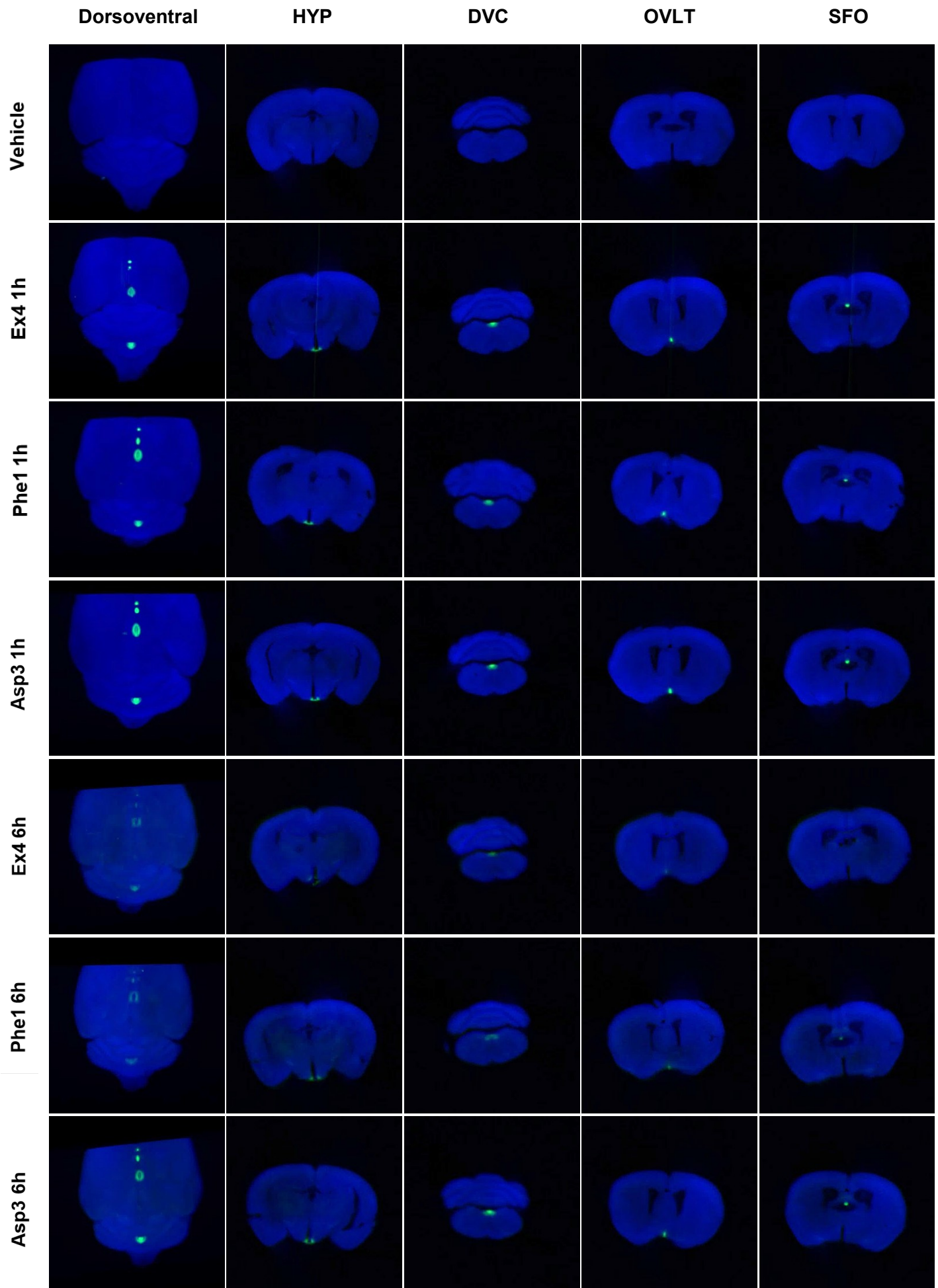


Figure 4.9. Distribution of sCy7-tagged GLP-1RAs in the mouse brain.

Representative maximum intensity projections of brains from vehicle or sCy7-tagged agonist-treated (100 nmol/kg) mice (n=2-3). Far left column shows the whole brain in the dorsoventral plane; columns to the right show coronal sections from (left to right) the HYP, DVC, OVLT and SFO. For all images, sCy7-tagged agonist signal from tissue excitation at 730 nm is shown in green; tissue autofluorescence from excitation at 470 nm is shown in blue as an anatomical reference. Imaging, processing and presentation settings were identical for all samples.

The regions in which agonist was detected were either circumventricular organs (CVOs: ME, AP, OVLT and SFO), or regions adjacent to circumventricular organs (ARC, NTS). The observed distribution pattern is a logical one, as the presence of fenestrated capillaries in CVOs make them entry points for blood borne substances, and adjacent regions would be the first point of spread after entry to the brain parenchyma. Importantly, all regions in which agonist was detected express the GLP-1R [197], and the ARC and NTS are critical neural substrates for the anorectic effects of GLP-1RAs [193].

Notably, the observed pattern of distribution was similar to that previously reported for fluorescently-tagged liraglutide [179], [427]. However, a more limited distribution was observed here, with signal absent in the PVN, DMH, posterior part of the periventricular nucleus of the hypothalamus (PVp), supraoptic nucleus (SO), tuberal nucleus (TU), and DMV. This may reflect genuine differences in agonist penetration due to differences in the properties of liraglutide *versus* exendin-4. For example, the longer circulatory half-life of liraglutide provides a more sustained level of drug exposure, allowing greater ingress into brain territories not labelled by the exendin-derived compounds used in the present study. Alternatively, it may represent a lack of imaging sensitivity or resolution in the analysis performed here. Salinas et al. used light sheet microscopy that is superior in imaging sensitivity and resolution to optical projection tomography, and also carried out co-registration of the Allen Mouse Brain Atlas to 3D brain images using a commercial software package not available in our unit, facilitating accurate identification and delineation of brain nuclei. It is conceivable that regions located adjacent to those where agonist was detected here – such as the DMV (adjacent to the AP and NTS; the three regions collectively comprise the DVC), or the PVp (adjacent to the ARC) – were labelled with agonist but were not successfully distinguished from neighbouring nuclei.

At one hour, exendin-4-pra12-sCy7 was present at slightly reduced levels relative to the other agonists in all brain regions, with hypothalamic differences being most significant (Figure 4.8 A). Agonist concentration in all brain regions decreased notably between 1- and 6-hour time points, likely reflecting renal clearance of the peptides over time. However, the reduction over time observed with exendin-asp3-pra12-sCy7 was less marked than with the other two compounds, with a greater total signal from exendin-asp3-pra12-sCy7 at 6 hours remaining in all brain regions.

These results can be examined in relation to the *in vitro* properties of the sCy7 conjugates highlighted above. The first point to observe is that at the 1-hour time-point, all compounds achieved similar total signal, albeit with a small reduction seen with exendin-4-pra12-sCy7 (Figure 4.8 A). In view of the marked differences in agonist potency (presumably reflecting, at least in part, reduced binding affinity), this suggests that the dose administered was sufficiently high to maximally label all accessible GLP-1Rs. Moreover, whilst receptors in the CVOs would be equally accessible to all compounds and thus signal from these regions is a useful marker of “total binding”, the spread of

fluorescent signal to ARC and NTS provides additional information about whether agonist pharmacological properties can affect their transport across the BBB/BCSFB. Whilst signal in the ARC vs. ME, and AP vs. NTS were not specifically quantified, all agonists occupied a broadly similar total volume at the 1-hour time point (Figure 4.8 B), suggesting matched ability to access the non-CVO regions. The key conclusion here is that the lack of a difference in access to protected brain areas by biased agonists with different endocytosis profiles argues *against* a specific GLP-1R-mediated transcytotic uptake mechanism of GLP-1RAs into the brain.

Nevertheless, some interesting conclusions can be drawn from comparison of the temporal changes in signal distribution with each agonist. In particular, at 6 hours, exendin-asp3-pra12-sCy7 was still present at approximately 50-100% of the levels seen at 1 hour, but very little exendin-4-pra12-sCy7 and exendin-phe1-pra12-sCy7 signal remained (Figure 4.8). Two possible explanations for this observation arise. Firstly, the significant differences in agonist binding affinity suggested by their discrepant cAMP signalling potencies are likely to determine the amount of ligand bound during dynamically changing circulating ligand concentrations. The half-life of exendin-4 in mice after intravenous injection is approximately 20 minutes [444] and slightly longer after subcutaneous administration due to the contribution of delayed absorption [445], with data from the latter study indicating circulating levels at 6 hours post injection can be >1000-fold lower than at 1 hour. Pharmacokinetic studies (Figure 3.2) indicate exendin-4, exendin-phe1 and exendin-asp3 are cleared at similar rates. The higher affinity of exendin-asp3-pra12-sCy7 would therefore be expected to result in a higher degree of ligand bound at this submaximal concentration after extensive clearance, with this being entirely compatible with the similar degree of binding at the supramaximal, saturating concentrations at the earlier time-point. Secondly, the more extensive ligand uptake of exendin-asp3-pra12-sCy7, at least compared to exendin-phe1-pra12-sCy7, and presumed slower recycling, should result in greater accumulation in GLP-1R-expressing neurons over time, thereby reflecting the ligand trafficking properties.

Interpreting these results in relation to the anorectic effects of unmodified exendin-4, exendin-phe1 and exendin-asp3 is more challenging. The lack correlation between apparent agonist brain concentration (both in CVOs accessible to the systemic circulation and in protected regions behind the BBB), anorectic effects and agonist trafficking profiles suggests that the entry of exendin-4-based agonists is not regulated by GLP-1R endocytosis at the BBB, and that the magnitude of agonist-induced anorexia is not explained by differences in brain entry. This lends support to the alternative hypothesis: that the divergence between glucoregulatory and anorectic effects of these biased agonists is due to tissue-specific differences in signalling between neurons and pancreatic β -cells.

4.3.5 *In vitro* signalling of VT750-tagged GLP-1RAs

Conjugation of sCy7 to the biased GLP-1RAs resulted in significant loss of signalling potency and anti-hyperglycaemic efficacy in a manner that did not affect all agonists equally, rendering these conjugates suboptimal as models of the unmodified agonists. Conjugation at pra12 to an alternative near-infrared fluorophore VivoTag-S 750 Fluorochrome (VT750), used in similar studies previously [179] was therefore tested. Contrasting with results using sCy7-conjugated agonists, VT750 was much better tolerated for cAMP signalling and GLP-1R internalisation (Figure 4.10, Table 4.4, Figure 4.11). A minor loss in cAMP potency was evident with exendin-phe1-pra12-VT750, which, together with the dramatic effect of sCy7 on the same base peptide and previous studies using exendin-phe1-

FITC [323], suggests that exendin-phe1 is more vulnerable to such changes than other ligands. In contrast, exendin-asp3-pra-12-VT750 appeared to be even more potent than exendin-asp3. In internalisation studies, signal from VT750-tagged agonist co-localised with the SNAP-tag labelled GLP-1R, supporting the agonist-dependent nature of receptor internalisation. Additionally, a VT750 tagged form of GLP-1R antagonist exendin(9-39) (exendin(9-39)-pra12-VT750) was synthesised for later studies. Consistent with unmodified exendin(9-39), exendin(9-39)-pra12-VT750 did not induce GLP-1R internalisation. As the molecular structure for VT750 is proprietary, it is difficult to posit the reason for the marked differences in effect between sCy7 and VT750 conjugation. However, it is evident that the latter does not sterically or electrostatically interfere with agonist-receptor binding or activation to the same extent as sCy7.

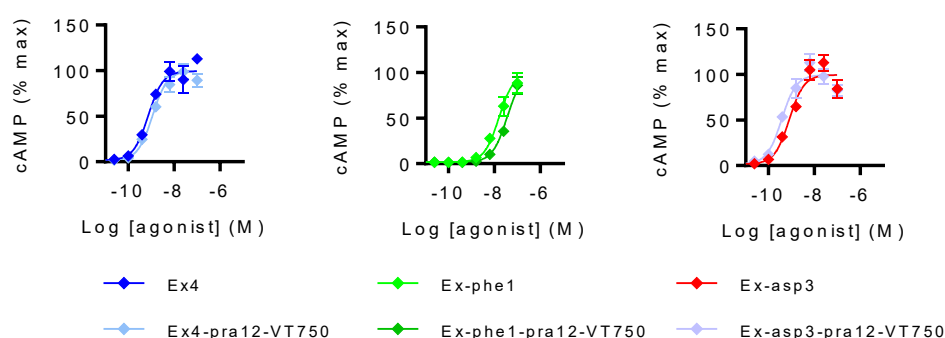


Figure 4.10. cAMP accumulation responses to VT750-tagged GLP-1RAs.

cAMP response to unmodified and VT750-tagged exendin-4, exendin-phe1 and exendin-asp3 in HEK293-SNAP-GLP-1R cells with four-parameter logistic fit of data, 30 min incubation.

Peptide	pEC ₅₀	ΔlogEC ₅₀	EC ₅₀ Ratio	E _{max} (%)	Ratio
Ex4	9.11 ± 0.04			105 ± 3.59	
Ex-phe1	7.76 ± 0.09			98.7 ± 12.1	
Ex-asp3	9.07 ± 0.03			107 ± 3.71	
Ex4-pra12-VT750	8.93 ± 0.05	0.18 ± 0.03	1.52	94.8 ± 4.76	0.90
Ex-phe1-pra12-VT750	7.43 ± 0.04	0.33 ± 0.05	2.12	102 ± 21.6	1.44
Ex-asp3-pra12-VT750	9.38 ± 0.08 *	-0.31 ± 0.03	0.49	95.3 ± 7.02	0.89

Table 4.4. Efficacy and potency for cAMP accumulation responses for VT750-tagged GLP-1RAs.

Values derived from data Figure 4.10. “ΔlogEC₅₀” is the difference in logEC₅₀ values between unmodified and VT750-modified counterparts. “EC₅₀ Ratio” describes the ratio between the EC₅₀ values for unmodified and VT750-modified counterparts. “Ratio” denotes E_{max} value relative to that of the equivalent unmodified compound. E_{max} values are normalised to global maximum in individual experiments. Significance reported in comparison to unmodified peptide e.g. ex4 vs. ex4-pra12-VT750. One-way randomised block ANOVA with Tukey's test. *p<0.05. Data reported as mean ± SEM from 4 independent experiments.

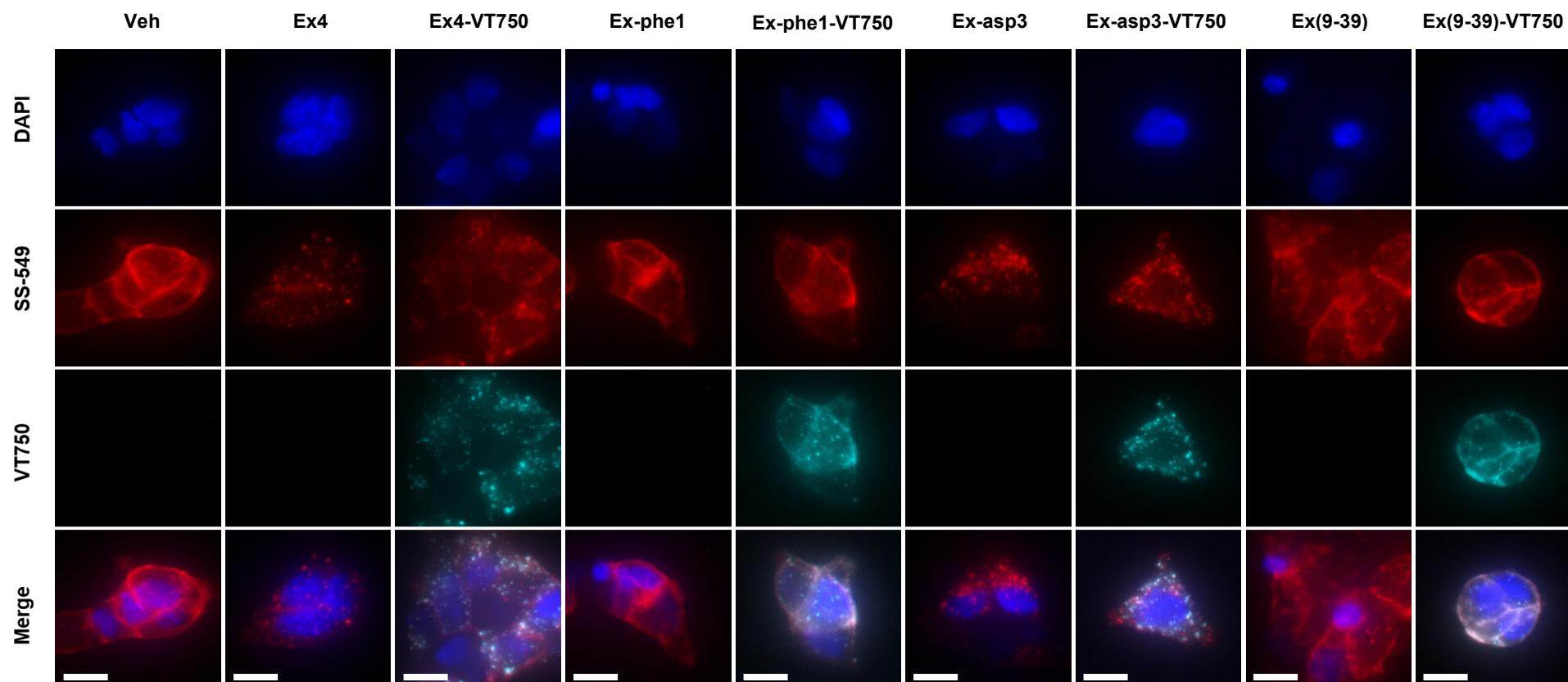


Figure 4.11. GLP-1R internalisation in response to VT750-tagged GLP-1RAs and exendin(9-39).

Representative images indicating internalisation of SNAP-GLP-1R labelled with SNAP-Surface 549 ("SS-549"; red) after 30 minutes of stimulation at 100 nM in HEK293-SNAP-GLP-1R cells. VT750 signal for VT750-tagged agonists shown in green. n=3 experiments, scale bar denotes 8 μ m.

4.3.6 Glycaemic responses to VT750-tagged GLP-1RAs

Testing of glycaemic responses in 6 hour delayed IPGTTs revealed exendin-4-pra12-VT750 and exendin-phe1-pra12-VT750 to induce similar and significant anti-hyperglycaemic effects, whereas exendin-asp3-pra12-VT750 produced weaker effects (Figure 4.12). These results are a marked improvement upon those at a matched dose for the sCy7-tagged agonists, where exendin-phe1-pra12-sCy7 failed to have any, and exendin-asp3-pra12-sCy7 produced the greatest anti-hyperglycaemic effect (i.e. sCy7 compounds showed the opposite pattern to that of non-fluorescent biased GLP-1RAs; Figure 4.6). However, the VT750-tagged agonists do not perfectly recapitulate the properties of the unmodified agonists, as exendin-phe1's usual enhanced effects relative to exendin-4 were not observed at the dose tested. It is conceivable that the seemingly minor impact on GLP-1R signalling of the VT750 conjugation in fact has real manifestations *in vivo*, e.g. the reduction in potency with exendin-phe1-pra12-VT750 may have negated its usual advantages at the single agonist dose tested during the IPGTT. Ideally, food intake studies would have been performed but the relatively small quantities of VT750 conjugates available meant that biodistribution studies were prioritised.

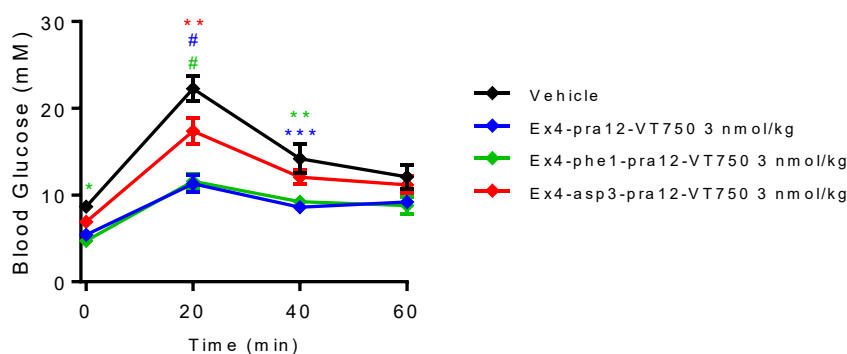


Figure 4.12. Glycaemic responses after administration of VT750-tagged GLP-1RAs.

Blood glucose during an IPGTT (2 g/kg glucose) in lean, male C57Bl/6 mice with glucose challenge 6 hours after intraperitoneal administration of VT750-tagged agonists at 3 nmol/kg, n=11. Two-way ANOVA with Tukey's test, with significance vs. saline shown. **p<0.01, ***p<0.001, #p<0.0001. Data represented as mean $\pm$ SEM.

4.3.7 Brain entry of VT750-tagged GLP-1RAs

Brain entry was assessed for the VT750-tagged agonists using the whole brain clearing method described above. As before, VT750-tagged agonist was detected in the same regions as for sCy7-tagged agonists: the ARC, ME, AP, NTS, OVLT and SFO. Quantification data are shown in Figure 4.13, magnified 3D projections of the HYP and DVC are shown in Figure 4.14, and representative images for each agonist at both time points are shown in Figure 4.15. Of note, the lower threshold used for the quantification of agonist signal sum and volume was 8000 intensity units in this experiment compared to 5000 units used for sCy7-tagged compounds. As before, this lower threshold was derived from vehicle-treated brains from the same experiment, and reflects a higher level of tissue autofluorescence in the samples of the VT750 experiment. Differences in background autofluorescence can be attributed to subtle differences in the clearing process (e.g. the age/degree of oxidation of reagents) and the use of older animals. The latter applies as the build-up of autofluorescent substances such as lipofuscin increases with age in tissues with a low rate of cell

turnover, such as the brain [446]. One exendin-phe1-pra12-VT750-treated 6-hour brain was excluded from quantification analysis due to interference from highly autofluorescent contaminants near the regions of interest.

As for sCy7 compounds, at one hour, exendin-phe1-pra12-VT750 was present at modestly higher total levels (Figure 4.13 A) than the other two agonists in most of the abovementioned brain regions, whilst levels for exendin-4-pra12-VT750 and exendin-asp3-pra12-VT750 were matched (Figure 4.13 A). At six hours, this pattern was reversed, with exendin-phe1-pra12-VT750 being present at significantly lower levels than the other two agonists for the majority of the regions of interest (Figure 4.13 A – with the exception of the SFO, where exendin-4-pra12-VT750 levels were lowest). The detection of exendin-4-pra12-VT750 at similar levels to exendin-asp3-pra12-VT750 at 6 hours (unlike in the sCy7 experiment, where exendin-4-pra12-sCy7 were much lower at this time point) likely reflects the better preservation of receptor binding affinity for exendin-4 (using *in vitro* signalling potency as a proxy) after VT750 *versus* sCy7 conjugation.

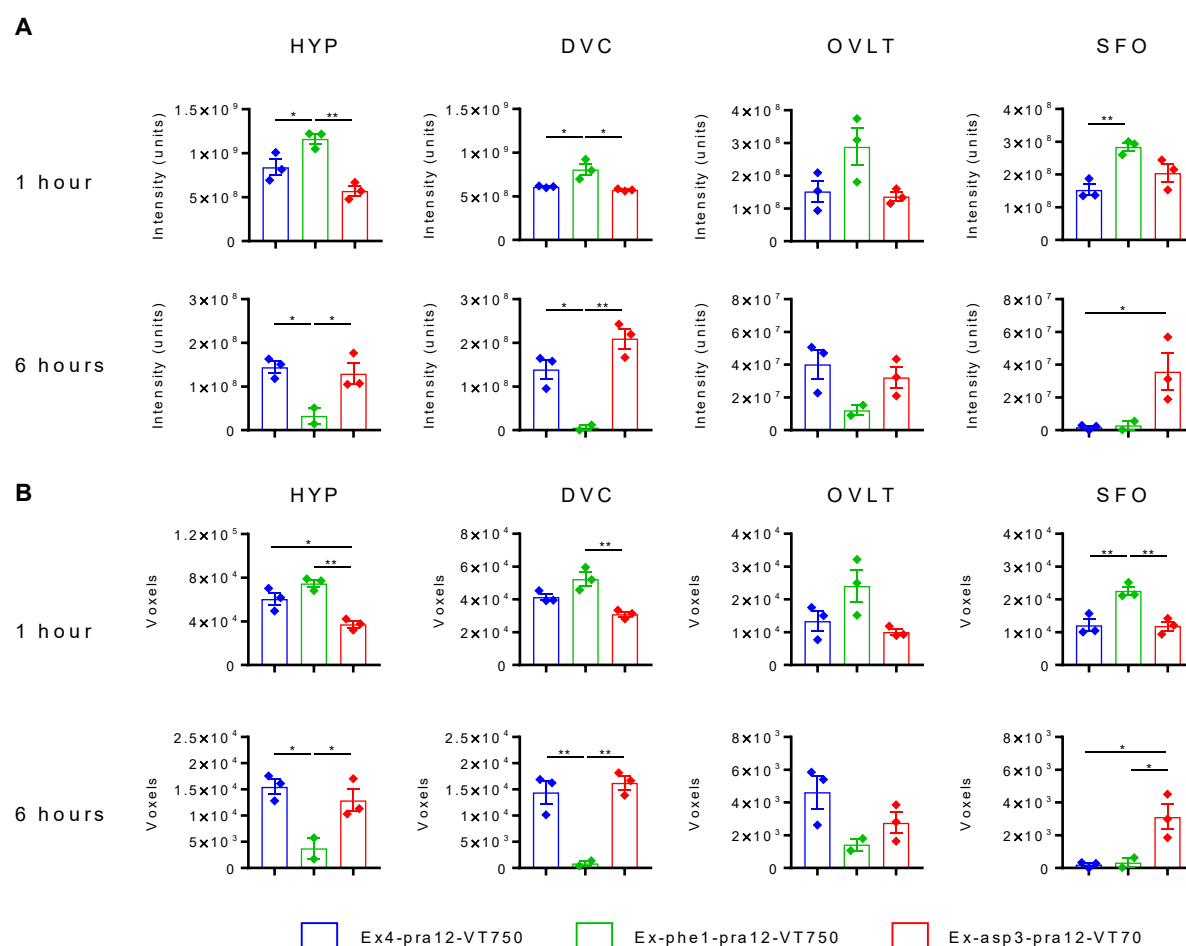


Figure 4.13. Quantification of VT750-tagged agonist levels and volume in individual brain regions.

(A) Total fluorescence signal from the VT750-tagged GLP-1RAs in the hypothalamus (ARC and ME), DVC (AP and NTS), OVLT and SFO at 1 and 6 hours post administration at 100 nmol/kg (n=2-3). Quantification was conducted using ImageJ by summing intensity values for all voxels within the region with an intensity value >8000 units. **(B)** As in (A) but reporting total volume of fluorescent signal. One-way ANOVA with Tukey's test. *p<0.05, **p<0.01. Data represented as mean ± SEM.

Again, whilst the quantification of hypothalamic and DVC regions performed here did not discriminate CVO-located signal (ME and AP) from that spreading beyond the BBB into the ARC and NTS, it is apparent from visual inspection of Figure 4.14 that exendin-phe1-pra12-VT750 was at least as abundant in the protected regions as the other two agonists. This pattern is supported by analysis of the volume or spread of agonist signal (i.e. above-threshold signal recognised as a discrete object through automated image analysis) in these regions as a surrogate for ARC and NTS encroachment. Thus, at one hour, exendin-4-pra12-VT750 and exendin-phe1-pra12-VT750 showed considerably greater spread than exendin-asp3-pra12-VT750 (Figure 4.13 B, Figure 4.14). This relative relationship between the biased GLP-1RAs would not be predicted by their respective endocytic properties if access to protected brain areas past CVOs was mediated by a GLP-1R-dependent transcytotic uptake mechanism. These data, together with the sCy7 experiment data, argue against a receptor-mediated mode of GLP-1RA brain uptake.

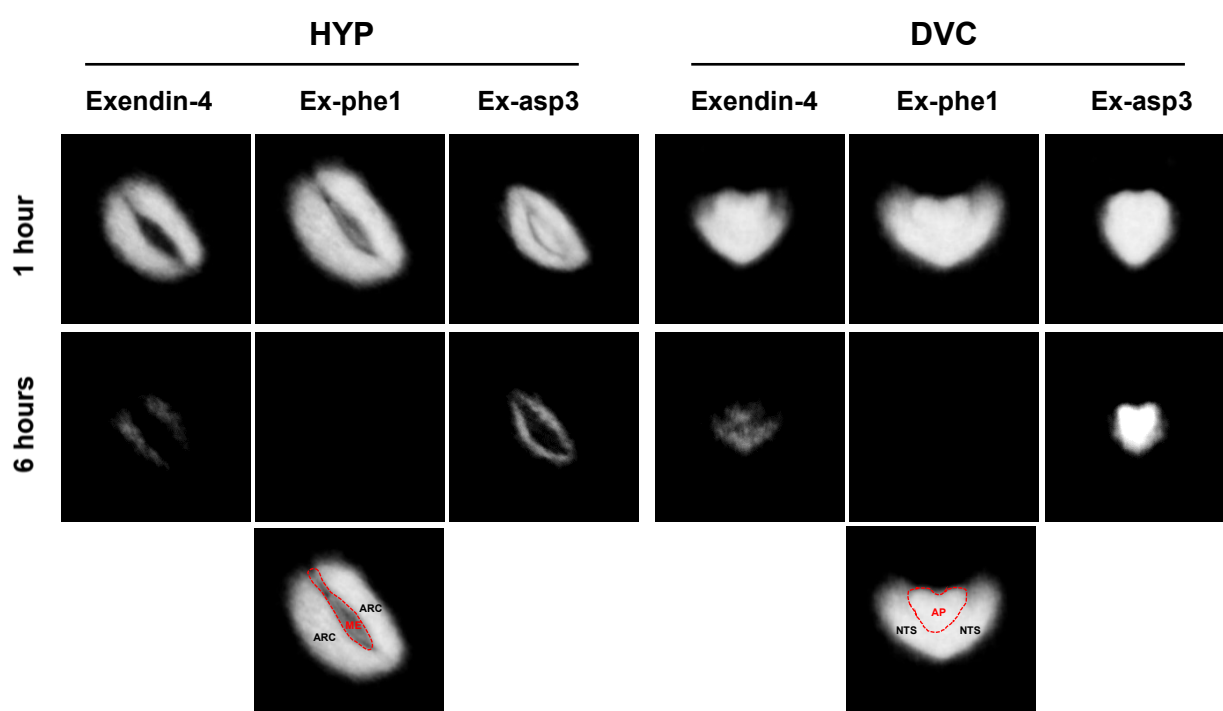


Figure 4.14. VT750-tagged agonist signal in the hypothalamus and dorsal vagal complex.

Representative 3D projections of fluorescent signal from VT750-tagged agonists in the hypothalamus (HYP) and dorsal vagal complex (DVC) at 1 and 6 hours post administration at 100 nmol/kg. Bottommost images denote the relative locations of the ME and ARC in the HYP, and AP and NTS in the DVC. Imaging, processing and presentation settings were identical for all samples.

Although access to barrier-protected regions appears to be independent of GLP-1R endocytosis, the relative trafficking profiles of the agonists seems to determine the degree of overall ligand spread. Exendin-asp3-pra12-VT750, a higher affinity GLP-1R ligand that exhibits more extensive endocytic uptake, might be predicted to be sequestered in neurons in CVOs and fail to travel extensively. Correspondingly, at 1 hour, exendin-asp3-pra12-VT750 shows lower levels in the ARC and NTS as compared to their respective CVOs the ME and AP. By contrast, low-internalising, and presumed fast-recycling exendin-phe1-pra12-VT750 is abundantly present in the ARC and NTS, suggestive of it

being rapidly released from neurons to which it first bound, and undergoing rounds of binding, uptake and release by neurons increasingly distant from the point of brain entry. Agonist levels at 6 hours supports this notion of relative agonist “mobility”, with exendin-phe1-pra12-VT750 being virtually undetectable in the HYP and DVC, suggesting spread out of these regions, whilst exendin-asp3-pra12-VT750 remained present, though at lower levels (for example, note the lack of exendin-asp3 in the ME at 6 hours in Figure 4.14).

Overall however, and corroborating the conclusion drawn from the sCy7 brain uptake experiment, the lack of correlation between biased GLP-1RA uptake into brain protected regions, trafficking profile and anorectic effects suggests a GLP-1R-independent uptake of GLP-1RAs, and that the degree of agonist-induced hypophagia is not explained by differential access to appetite-regulating centres.

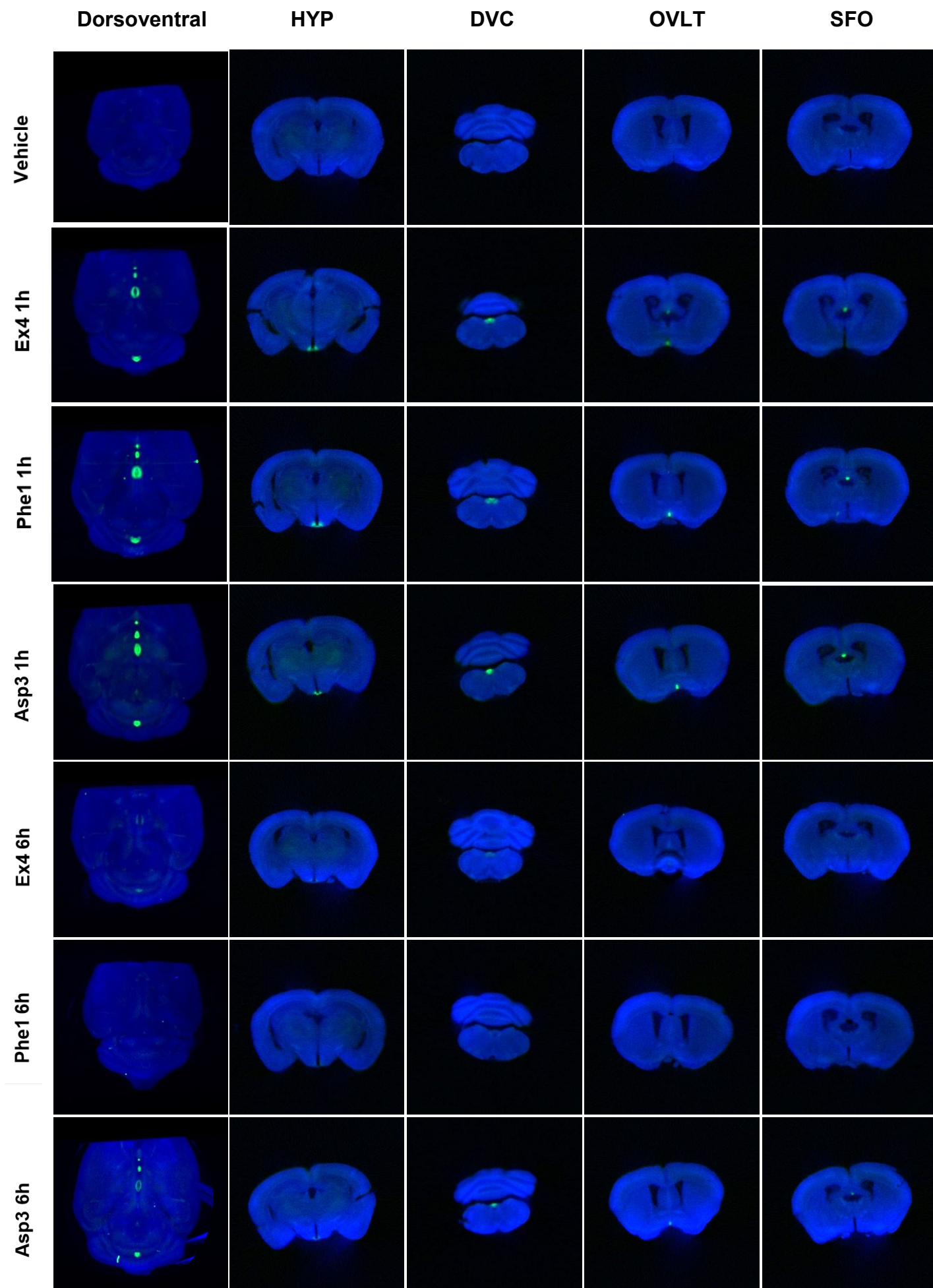


Figure 4.15. Distribution of VT750-tagged GLP-1RAs in the mouse brain.

Representative maximum intensity projections of brains from vehicle or VT750-tagged agonist-treated (100 nmol/kg) mice (n=2-3). Far left column shows the whole brain in the dorsoventral plane; columns to the right show coronal sections from (left to right) the HYP, DVC, OVLT and SFO. For all images, VT750-tagged agonist signal from tissue excitation at 730 nm is shown in green; tissue autofluorescence from excitation at 470 nm is shown in blue as an anatomical reference. Imaging, processing and presentation settings were identical for all samples.

4.3.8 Pancreatic levels of VT750-tagged biased GLP-1RAs

Pancreata from mice administered VT750-tagged agonists were also collected in parallel to brain specimens (Figure 4.13, Figure 4.15) and cleared to allow assessment of agonist levels at β -cells. As well as being directly relevant to the glucoregulatory effects of biased GLP-1RAs, these data provide a reference point against which brain uptake data can be compared to support or refute agonist-specific differences in access to protected brain regions as islets are directly accessible to agonist in the systemic circulation. At one hour, VT750-agonist signal was clearly visible in islet-like structures (Figure 4.16). On this occasion signal was not quantified, but visually there was a trend (3 pancreata examined per treatment) towards increased uptake with exendin-phe1-pra12-VT750 at this time-point, in keeping with the modestly increased signal in the brain. The concordance between agonist levels in pancreas and in protected brain regions again argues against ligand-specific differences in BBB penetration. Notably, at 6 hours, the rank order of pancreatic concentration was (from highest to lowest) exendin-asp3-pra12-VT750, exendin-4-pra12-VT750 and exendin-phe1-pra12-VT750 (Figure 4.16), thus matching the pattern of results seen in the brain.

It should be noted that the observed fluorescent signal is likely derived from both receptor-agonist complex located at the cell surface as well as that that has internalised. The lack of cellular resolution in these studies prevents direct assessment of this, but the presence of signal from non-internalising exendin(9-39)-VT750 (see Figure 4.21, i.e. this signal is membrane-derived) and from previous studies showing internalisation of Alexa Fluor 594-tagged liraglutide in pancreatic slices [179] suggest that both fractions contribute. Bearing this in mind, the 6-hour observations correspond with the agonists' trafficking profiles, with endocytosis-prone exendin-asp3 showing stronger signal due to intracellular sequestration of receptor-agonist complex, whereas endocytosis-deficient exendin-phe1 shows low signal as cell surface-located receptor (which constitutes the major receptor fraction for exendin-phe1-treated cells) is bound and subsequently unbound by agonist relatively rapidly (due to exendin-phe1's low residence time).

Of note, agonist signal was observed in extra-islet, tubular structures resembling vasculature or ducts in only exendin-phe1-pra12-VT750 pancreata at the one hour time point. This is unlikely to be non-specific binding given that it was observed in all three exendin-phe1-pra12-VT750-treated pancreata, and there is no reason to suspect non-specific binding of exendin-phe1-pra12-VT750 should differ from the other VT750 conjugates. The extra-islet signal thus appears to be a GLP-1R-dependent property of exendin-phe1-pra12-VT750 binding. What these structures represent is unclear, but could be interrogated with co-staining for markers of vasculature (e.g. platelet adhesion cell adhesion molecule 1, PECAM-1) or pancreatic exocrine tissue (e.g. carboxypeptidase A, Cpa1). However, this would not be trivial, as the clearing process used here is not optimised for immunostaining, and though a number of immunostaining-optimised protocols exist (e.g. iDISCO [447], CLARITY [448],

Cubic [449]), antibody optimisation must be conducted and the overall process is considerably protracted relative to the present protocol. Alternatively for blood vessels, administration of fluorescently tagged dextran or lectin may allow visualisation of the vasculature, as has recently been described for optically cleared pancreata [450]. Although the extra-islet signal resembles pancreatic vasculature in optically cleared specimens [450], [451], whether the GLP-1R is expressed upon vascular endothelial cells is, as yet, unclear [452], [453]. GLP-1R expression has been described in exocrine acinar cells however [142], [454].

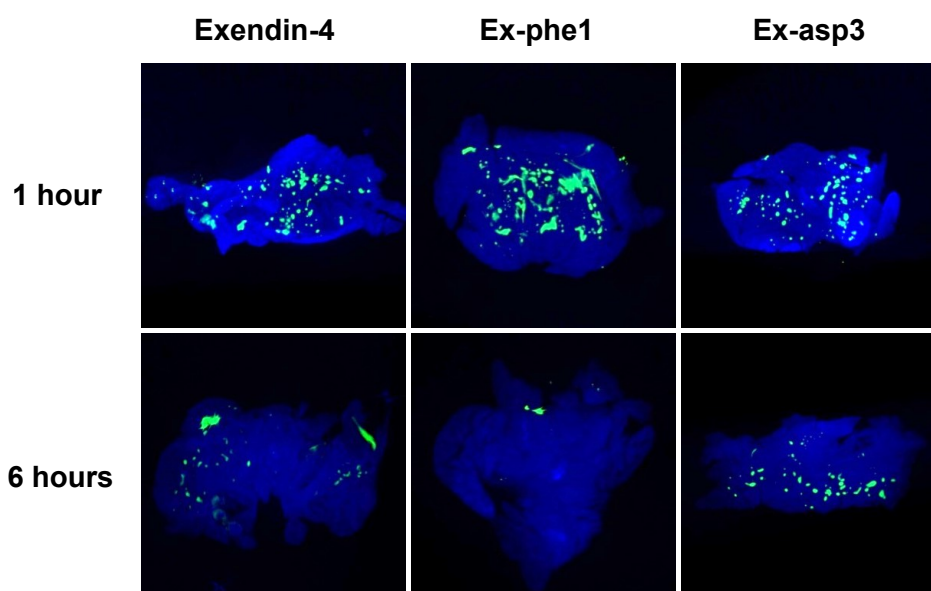


Figure 4.16. Pancreatic levels of VT750-tagged GLP-1RAs.

Representative projections of pancreas from vehicle or VT750-tagged agonist-treated (100 nmol/kg) mice (n=3). For all images, VT750-tagged agonist signal from tissue excitation at 730 nm is shown in green; tissue autofluorescence from excitation at 470 nm is shown in blue as an anatomical reference. Imaging, processing and presentation settings were identical for all samples. Large, bright green objects in the 6 hour pancreata for exendin-4 and exendin-phe1 represent contaminants as opposed to islets.

4.3.9 Measurement of cerebrospinal fluid concentrations of exendin-4 and exendin(9-39)

The results above demonstrate that bias-related differences in endocytosis do not lead to differences in CNS entry, and thus suggest that CNS entry of exendin-4-based GLP-1RAs is not reliant upon GLP-1R internalisation. However, they do not definitively exclude this possibility. To further assess the requirement for GLP-1R endocytosis in brain uptake, the GLP-1R antagonist exendin(9-39) was used as an “extreme” example of a non-internalising ligand (note that endocytosis with exendin-phe1 is slow but not insignificant) in comparison to exendin-4, which internalises rapidly. Here, rats were administered unmodified exendin-4 or exendin(9-39) at 100 nmol/kg, followed by CSF collection by puncture of the cisterna magna 1 hour later. Rats were used for this study to ensure sufficient CSF could be obtained for the sample requirements of the exendin ELISA. Peripheral blood was also taken by cardiac puncture, and peptide concentration in both fluid types measured. Results are summarised in Figure 4.17. Critically, CSF concentrations of exendin-4 and exendin(9-39) were matched, suggesting that brain entry of exendin-4-based GLP-1R ligands is not dependent upon receptor

endocytosis at the BBB (Figure 4.17 A). Similarly, no difference was seen in plasma concentration of the peptides at 1 hour (Figure 4.17 B). CSF levels of peptide were, on average, 3.79% of the plasma concentration, suggesting that only a small fraction of circulating peptide crosses the BBB and enters the CSF (Figure 4.17 C). It should be noted that the measured CSF concentration may not be representative of other brain areas. For example, local concentration in brain parenchyma in CVOs or regions proximal to CVOs may be significantly higher than that once it has been distributed throughout the CSF.

Brain entry of exendin-4 has been shown to be influenced by prevailing peptide concentration, with entry potentially being limited at high doses [423]. Thus to assess whether the results obtained were generalizable to lower, more physiological doses, the experiment was repeated with a 10 nmol/kg dose of exendin-4 or exendin(9-39). However, CSF peptide concentrations were at the lower detection limit of the ELISA used for measurement, precluding any accurate conclusions (data not shown).

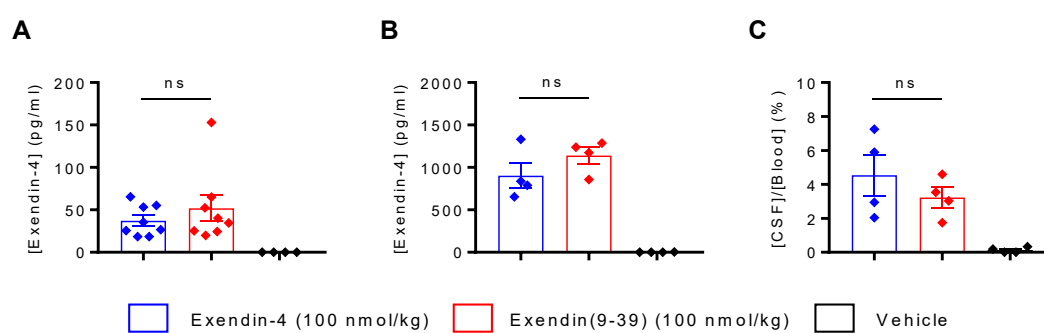


Figure 4.17. CSF and plasma concentration after peripheral administration of exendin-4 and exendin(9-39) in Wistar rats.

(A) CSF peptide concentration 1 hour after intraperitoneal administration of 100 nmol/kg of exendin-4 or exendin(9-39), or vehicle, n=8 except vehicle where n=4. (B) As in (A) but plasma peptide concentration, n=4. (C) CSF peptide concentration expressed as a percentage of plasma peptide concentration, as derived from data in (A) and (B). CSF and plasma readings are unpaired. Unpaired, two-tailed student's t-test. *p<0.05. Data represented as mean ± SEM.

4.3.10 Brain and pancreatic distributions of VT750-tagged exendin-4 and exendin(9-39)

To corroborate the results obtained from the measurement of CSF concentrations of exendin-4 and exendin(9-39), brain clearing experiments were conducted comparing the brain and pancreatic levels of VT750-tagged exendin-4 and exendin(9-39) after peripheral administration at 100 nmol/kg. In parallel, two lower doses of exendin-4-pra12-VT750 were tested. The rationale for this dose response assessment is as follows: the lack of differences in brain entry of the biased GLP-1RAs points away from agonist-specific differences in access to anorectic brain regions as the explanation for their divergent physiological effects. The favoured alternative explanation concerns differences between how neuron and β -cells respond to biased ligands (tissue bias). However, a further option is that each agonist has equal access to the brain, but CNS levels after peripheral administration of even pharmacologically relevant doses are universally lower than in the plasma and insufficient to cause significant levels of receptor desensitisation. The pancreas, by contrast, is exposed to high circulating

levels of agonist. Thus, only at the latter organ is exendin-phe1's reduced, and exendin-asp3's increased propensity to cause GLP-1R desensitisation manifested. This hypothesis can be interrogated by comparing agonist "concentration" between the two organs after peripheral administration. If the brain were less accessible than the pancreas, it would be predicted that for a range of lower doses, agonist would be present in the pancreas but absent in the brain. The results from the CSF collection study above lend support to this explanation, as levels of exendin-4 or exendin(9-39) in the CSF one hour after peripheral administration were only 3.8% of circulating levels. In addition, the requirement for notably higher peripheral doses of agonist to elicit anorectic *versus* glycaemic effects (Figure 3.4, Table 3.1) also supports this reasoning.

Results are summarised in Figure 4.18, Figure 4.19, Figure 4.20 and Figure 4.21. Firstly, no significant differences were seen in the overall brain signal of exendin-4-pra12-VT750 and exendin(9-39)-pra12-VT750 at equivalent doses (Figure 4.18 A, C), supporting results obtained in the CSF collection study. Interestingly, though no significant differences in signal volume were detected (Figure 4.18 B, C), the pattern of spread appeared subtly different, as best captured in Figure 4.20. Exendin(9-39)-pra12-VT750 appeared to show greater spread in the ARC and NTS relative to exendin-4, at least in comparison to the corresponding lower levels in seen CVOs the ME and AP. This mirrors the pattern seen between internalisation-deficient exendin-phe1 and internalisation-prone exendin-asp3, and thus may be attributable to their different endocytic profiles. Unfortunately, as before, the inability to quantify signal separately between the ME and ARC, and AP and NTS precludes direct comparison of peptide levels in the CVOs vs. CVO-adjacent nuclei.

Albeit insignificant, differences were observed in the pancreatic levels of exendin-4-pra12-VT750 and exendin(9-39)-pra12-VT750, with the latter being present at lower levels (Figure 4.18 D). This may reflect the fact that exendin(9-39)-pra12-VT750 signal derives only from agonist-bound receptor at the membrane (given a lack of receptor internalisation), whereas exendin-4 signal likely includes contributions from both membrane and cytoplasmic fractions. Additionally, exendin(9-39)-pra12-VT750, but never exendin-4-pra12-VT750 signal was detected in extra-islet structures resembling the vasculature. Given a similar observation for exendin-phe1-pra12-VT750-treated pancreata (Figure 4.16), this may be a property related to internalisation-deficient GLP-1R ligands.

Regarding the dose response assessment for exendin-4, the results suggest that differences do not exist in agonist access between the brain and the pancreas. Whilst exendin-4 was undetectable in both organs at 1 nmol/kg, it was detected at low levels in both organs at 10 nmol/kg, and at high levels at 100 nmol/kg (Figure 4.18 C & D). An approximate measure of agonist "concentration" at the two organs was determined by dividing the total intensity values by the total volume values (reported as "mean", Figure 4.18 E). Agonist "concentration" in the brain was approximately 5-fold higher than in the pancreas. This measure comes again with two caveats relevant to its interpretation. Firstly, it is presumably dependent partly on GLP-1R density, as only ligand that has been locally concentrated by the formation of ligand-receptor complexes and/or endocytic uptake will be detected. Secondly, as before, the quantification includes contributions from CVOs that have free access to systemic ligand; however, using the HYP region shown in Figure 4.20 as an example, anatomically plausible signal originating from the ring-like ARC structure can be seen after 10 nmol/kg agonist dosing. Thus, the exclusion of this hypothesis points towards differences in the direct action of the biased agonists upon neurons and pancreatic β -cells underlying the divergence between glucoregulatory and anorectic effects.

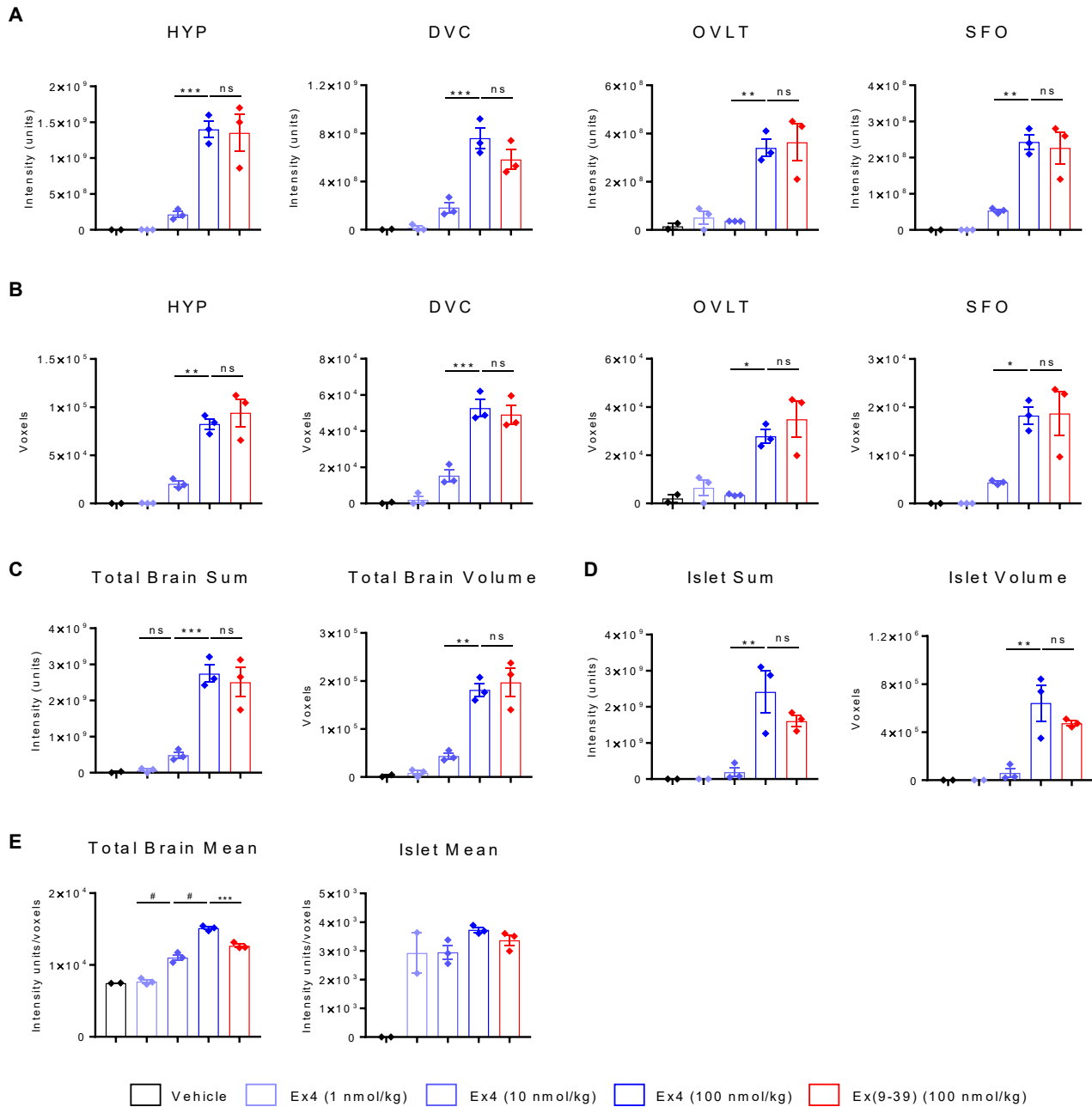


Figure 4.18. Quantification of VT750-tagged peptide concentration in the brain and pancreas.

(A) Total fluorescence signal from the VT750-tagged exendin-4 and exendin(9-39) in the hypothalamus (ARC and ME), DVC (AP and NTS), OVLT and SFO at 1 hour post administration at the indicated doses (n=3). Quantification was conducted using ImageJ by summing intensity values for all voxels within the region with an intensity value >7000 units. **(B)** As in (A) but reporting total volume of fluorescent signal. **(C)** Total brain signal intensity or volume as determined by summing values from individual brain regions in (A) and (B) per brain. **(D)** Islet signal intensity and volume. Quantification was conducted as in (A) and (B) but with a lower intensity threshold of 2000 units and a lower size threshold of 50 voxels. Extra-islet structures observed in exendin(9-39)-pra12-VT750-treated pancreata were excluded from quantification. **(E)** Total brain and islet mean or “concentration” values, as determined by intensity/volume using data from (A)-(C). One-way ANOVA with Tukey’s test. *p<0.05, **p<0.01, ***p<0.001. Data represented as mean ± SEM.

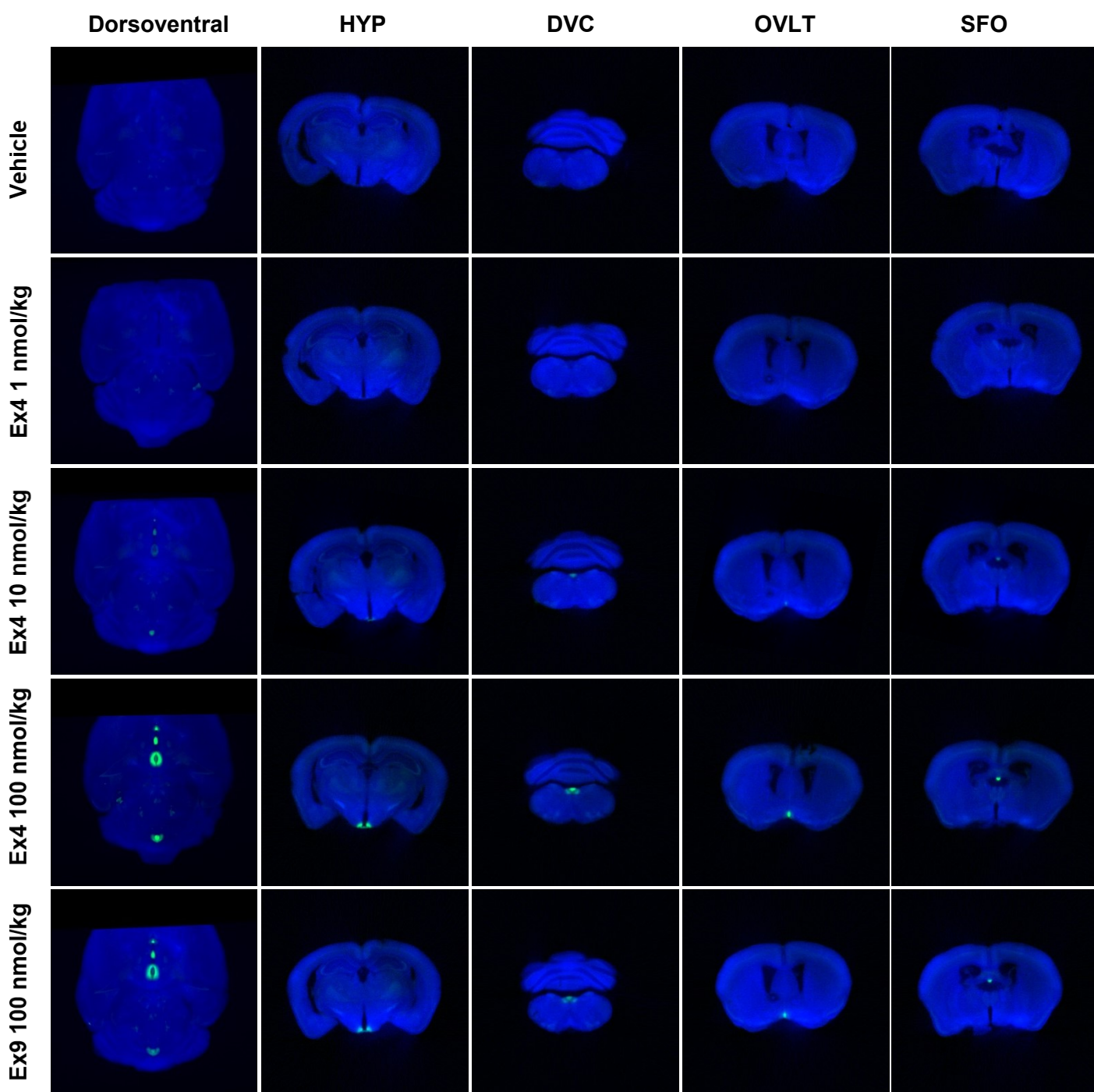


Figure 4.19. Distribution of VT750-tagged exendin-4 and exendin(9-39) in the mouse brain.

Representative maximum intensity projections of brains from mice treated with vehicle, or exendin-4-pra12-VT750 or exendin(9-39)-pra12-VT750 at the indicated doses (n=3). Far left column shows the whole brain in the dorsoventral plane; columns to the right show coronal sections from (left to right) the HYP, DVC, OVLT and SFO. For all images, VT750-tagged peptide signal from tissue excitation at 730 nm is shown in green; tissue autofluorescence from excitation at 470 nm is shown in blue as an anatomical reference. Imaging, processing and presentation settings were identical for all samples.

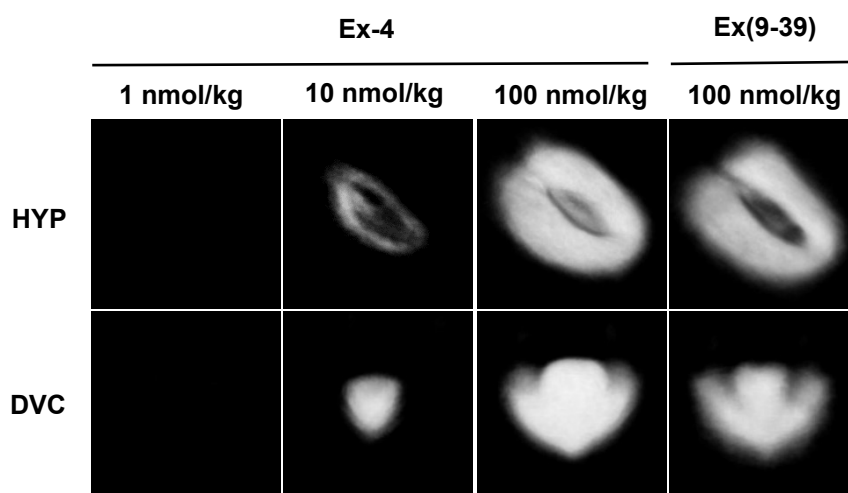


Figure 4.20. VT750-tagged exendin-4 and exendin(9-39) signal in the hypothalamus and dorsal vagal complex.

Representative 3D projections of fluorescent signal from exendin-4-pra12-VT750 and exendin(9-39)-pra12-VT750 in the hypothalamus (HYP) and dorsal vagal complex (DVC) at 1 hour post administration at the indicated doses. Imaging, processing and presentation settings were identical for all samples.

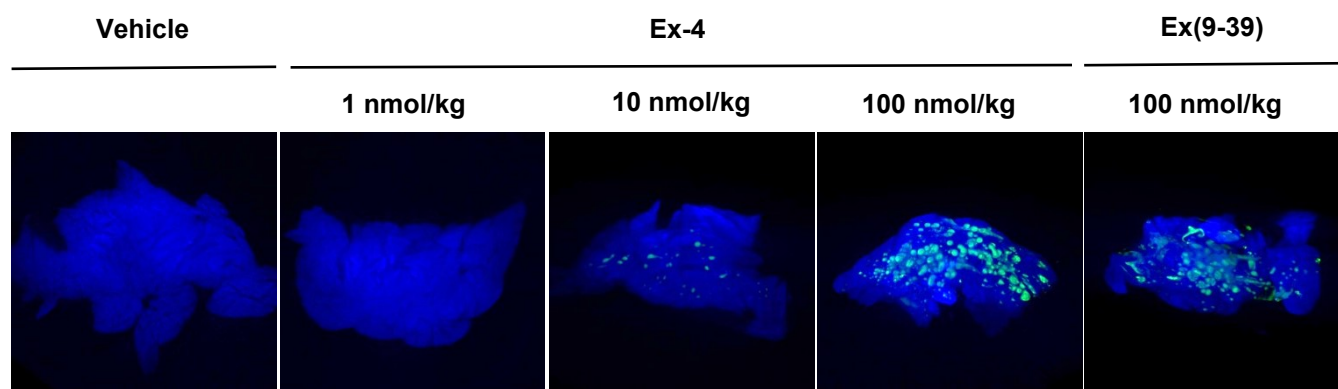


Figure 4.21. Pancreatic levels of VT750-tagged exendin-4 and exendin(9-39).

Representative projections of pancreas from mice treated with vehicle, or exendin-4-pra12-VT750 or exendin(9-39)-pra12-VT750 at the indicated doses (n=3). For all images, VT750-tagged peptide signal from tissue excitation at 730 nm is shown in green; tissue autofluorescence from excitation at 470 nm is shown in blue as an anatomical reference. Imaging, processing and presentation settings were identical for all samples.

4.4 Discussion

This chapter interrogated the hypothesis that the loss of bias-related effects upon appetite suppression – specifically the time-dependent signalling benefit of exendin-phe1, and deficit of exendin-asp3 – was due to differential agonist access to appetite-regulating nuclei in the CNS. The key findings can be summarised as follows:

1. Overall GLP-1R ligand access to CNS sites was similar to the pancreas.
2. Access to CNS neurons was similar for each agonist.
3. Spread beyond the BBB from CVOs to ARC and NTS, or into the CSF, was not reduced with ligands showing decreased GLP-1R endocytosis, arguing against the latter as an important mechanism for transfer of GLP-1R ligands into protected brain areas.
4. Some differences were observed with ligand spread within the brain parenchyma, with a more diffuse distribution noted for ligands with reduced endocytosis.
5. Disappearance of ligand from both brain and pancreas over time was correlated with the ligand endocytosis rate, with fluorescent signal from fast-internalising exendin-asp3-VT750 remaining detectable for longer.

4.4.1 CNS and pancreas access is equal for GLP-1R ligands

Evidence for the first point rests on comparison of average fluorescence intensities quantified from exendin-4-VT750-positive brain regions and pancreatic islets, which were broadly similar (Figure 4.18). At first glance this contrasts with the fact that CSF concentrations of exendin-4 (and indeed, exendin[9-39]) were considerably lower than in plasma (Figure 4.17), in keeping with prior reports [179], [428], [455]. However, the two observations are mutually compatible as agonist could be both concentrated at GLP-1Rs at its site of action whilst also being diffusely distributed throughout the CSF. As agonist-related homologous desensitisation tends to be most apparent at high doses, the finding of similar ligand concentrations in brain *versus* pancreas is important as it excludes this as a factor influencing potential for bias-related desensitisation.

4.4.2 Uptake of GLP-1R ligands past the BBB is independent of GLP-1R endocytosis

Moreover, the results presented evidence penetration of exendin-4-based GLP-1RAs beyond the BBB to be independent of GLP-1R endocytosis. As an “extreme” example of a GLP-1R ligand with reduced endocytosis, the antagonist exendin(9-39) was detected at equivalent levels to exendin-4 in both the CSF and, as their VT750-tagged peptide conjugates, in the ARC and NTS. Thus, GLP-1R endocytosis does not appear to be required for ligand uptake across the BBB. This finding matches previous reports of exendin-4 crossing the BBB with rapid and non-saturable kinetics, suggesting a lack of receptor- or carrier-mediated transport [423]. However, it refutes a more recent hypothesis of GLP-1R-mediated brain uptake of liraglutide by tanycytes present in CVOs [179] akin to that demonstrated for leptin [391]; however no direct evidence for this mechanism for GLP-1RAs currently exists.

4.4.3 GLP-1R ligand spread within the parenchyma is related to their endocytic properties

In contrast to overall agonist levels, the extent of agonist spread within the protected brain areas (ARC and NTS) appeared to correlate with their endocytic properties. Specifically, an inverse relationship was observed between the degree of agonist-induced internalisation and apparent diffusion within these areas over time, with exendin-phe1 showing the greatest, and exendin-asp3-VT750 the least amount of spread (Figure 4.13, Figure 4.14). Notably, this relationship extended to antagonist exendin(9-39)-VT750 (Figure 4.20), with its distribution pattern emulating that of exendin-phe1, and was also observed in the pancreas (Figure 4.16). This pattern is suggestive of internalisation-prone GLP-1R ligands being sequestered in GLP-1R-expressing cells in CVOs and nearby ARC/NTS neurons close to the site of entry, whilst internalisation-deficient ligands are capable of undergoing rounds of receptor dissociation and diffusion further into the brain. Perhaps related to the same phenomenon, disappearance of exendin-phe1-VT750 from brain parenchyma over time was faster than for exendin-asp3-VT750.

Though interesting, the physiological impacts of this differential spread are unclear, given that the anorectic effects of the agonists are matched over time. Of note, GLP-1R agonism in the nucleus accumbens, ventral tegmental area and hippocampus – brain regions more distant from CVOs – has been shown to reduce the rewarding properties of food, and induce hypophagia without nausea [219], [221]. It is conceivable that greater CSF-borne spread of exendin-phe1 leads to greater activation of these regions, potentially explaining its reduced induction of nausea-related behaviours relative to exendin-4 [323]. The fact that specific VT750 signal was not seen in these regions does not necessarily exclude this possibility, as the sensitivity of the utilised imaging approach is limited, as suggested by the fact that a dose of 1 nmol/kg exendin-4 results in robust anti-hyperglycaemic effect but the fluorescent ligand was undetectable in the pancreas at this level. Thus whilst overall appetite suppression may be matched, the nature of biased agonist-induced hypophagia may be qualitatively distinct (homeostatic, hedonic, nausea-related). To assess this, more rigorous assessments of nausea and reward-motivated responding after administration of the biased agonists (including exendin-asp3) could be conducted.

4.4.4 GLP-1RA-targeted brain regions vs. GLP-1R expression in the brain

The regions in which ligand was detected after peripheral administration was strikingly consistent: CVOs the ME, AP, SFO and OVLT, and CVO-adjacent regions the ARC and NTS. Over time, signal diminution in these regions was observed, but agonist was not detected in additional areas of the brain. The GLP-1R is expressed widely in the brain [91], [197] and thus the absence of agonist in other locations calls into question their necessity for GLP-1RA-mediated hypophagia. A few explanations can be offered as to the highly localised nature of agonist signal.

- Firstly, it may be the case that only limited regions of the GLP-1R-expressing brain are required to exert the anorectic effects of long-acting GLP-1RAs, as their improved pharmacokinetics likely results in supraphysiological activation of these regions. Indeed, ARC GLP-1Rs are sufficient to mediate exendin-4-induced hypophagia, as evidenced by intra-ARC administration (0.6 pmol/mouse) [210].

- Secondly, agonist may reach regions outside those where signal is detected, but levels may be below the detection limit of the OPT system being used, as discussed above. The use of superior light-sheet microscopy to detect VT750-tagged liraglutide reports a much more extensive distribution pattern than for the biased GLP-1RAs [427]; however, this may reflect genuine differences in the spread of exendin-4 and liraglutide due to agonist pharmacokinetic differences, rather than imaging resolution (as discussed in Section 4.3.4). The presence of exendin-4 in the CSF after peripheral administration (Figure 4.17) opens up the possibility of other regions being accessed by volume transmission, as has been suggested for endogenous GLP-1's effects upon the hippocampus [221], and for leptin [391].
- Thirdly, other GLP-1R-expressing locations may not be directly engaged by peripherally administered GLP-1RAs, but secondary activation of these regions may occur. Indeed, the majority of c-fos positive regions after peripheral liraglutide administration were not those directly bound by liraglutide-VT750; strong co-localisation of agonist and c-fos was only observed in the AP, ARC and OVLT [427]. However, secondary activation may not be GLP-1-mediated, as GLP-1-producing PPG neurons do not express the GLP-1R [200], [201] and thus cannot be directly activated by GLP-1RAs. PPG neurons could conceivably be part of the circuit of secondary activation, leading to "tertiary" activation of GLP-1R-expressing, PPG neuron-innervated locations.

Thus, a number of explanations may explain the disparity between GLP-1R-expressing regions and areas where fluorescent agonist is detected. However, an altogether different interpretation of the highly localised fluorescent signal is that it does not reflect "true" passage across the BBB. The majority of the regions in which agonist is detected are superficial CVOs (AP, ME, SFO, OVLT) [396], and under certain conditions the ARC is directly supplied by fenestrated capillaries [405]. Fluorescent signal may therefore reflect sequestration of agonist in blood-penetrable regions. A number of lines of evidence suggest that this is not the case. Firstly, the ventromedial ARC only appears to have direct privilege to blood-borne factors after prolonged (24-hour) fasting [405]; mice in the experiments conducted here remained fed, suggesting that the robust signal detected throughout the ARC was as a result of spread from the ME. Secondly, exendin-4 and exendin(9-39) were reliably detected in the CSF after peripheral administration, indicating penetration past CVOs and/or the BBB. Thirdly, distinct signal was detected in the NTS, and whilst the NTS (like the ARC) has been proposed to have partial CVO properties [456], strong evidence suggests otherwise [457] and thus the NTS is not considered a CVO [396]. Therefore, it is more likely that agonist presence within the ARC and NTS is the result of genuine spread from entry at adjacent CVOs.

4.4.5 The mechanism of GLP-1R ligand brain uptake

Accepting this, the results here provide further, direct evidence of GLP-1RA entry into the brain to add to the existing literature [179], [423], [427]. They thus corroborate studies demonstrating GLP-1R-expressing neurons in the CNS as the primary mediators of GLP-1RA-induced hypophagia [163], [180]. Understanding the site of anorectic action of GLP-1RAs is of renewed interest given the recent licensing of liraglutide as an anti-obesity medication. However, an equally important question to whether GLP-1RAs achieve brain uptake is the mechanism by which it occurs. The present results exclude a receptor-mediated mechanism based upon GLP-1R endocytosis. Alternative mechanisms for BBB and BCSFB crossing include diffusion, carrier-mediated transport (CMT), and adsorptive-

mediated transcytosis (AMT); existing data suggests that uptake occurs via diffusion, at least for exendin-4. Kastin and colleagues – who have pioneered research into the brain entry of peptides – previously reported brain uptake of exendin-4 and a degradation-resistant form of GLP-1 ([Ser8]GLP-1) to exhibit non-saturable kinetics characteristic of diffusion [194], [423]. Consistent with data here, they conclude brain uptake of [Ser8]GLP-1 to be GLP-1R-independent, as co-administration of exendin(9-39) failed to affect uptake kinetics [194]. However, they report exendin-4 uptake to be independent of CVOs [423], whilst the strong presence of fluorescently-labelled exendin-4 in CVOs reported here suggest that they do serve as an entry point.

Multi-ligand receptor LRP2 has been shown to transport insulin growth factor-1 [416] and leptin [417] across the BCSFB, raising the possibility that LRP2, or another “promiscuous” receptor facilitates the uptake of GLP-1R ligands. Provided that the differential endocytic properties of the biased GLP-1RAs are specific to the GLP-1R, this would be an alternative receptor-mediated mechanism consistent with the matched brain uptake of the agonists. However, uptake via this means would still exhibit rate-limiting kinetics, and is therefore not compatible with the existing literature [423]. By this same reasoning, CMT or AMT - other brain uptake mechanisms that would not differentiate between the endocytic properties of the agonists – can both be excluded, given that they also display saturable kinetics.

It should be noted that the molecular weight of exendin-4 (4187 Da) is over four times higher than the optimal molecular weight (<500 Da) for passive brain entry [390]. However, it possesses other, more important physicochemical properties (high lipophilicity and low hydrogen bonding) required for diffusion [423]. From their detailed study of numerous peptides, Kastin and colleagues conclude lipophilicity to be the most important determinant of transcellular BBB penetration [435], [458], and have reported non-saturable brain entry of endogenous, satiety-related peptides with similar or larger molecular weight to exendin-4, including NPY [432], nesfatin [433] and fibroblast growth factor 21 (FGF21) [434]. Thus, the data presented in this chapter and past studies point towards the brain uptake of exendin-4-based GLP-1R ligands to be a passive process. However, direct demonstration of this process by cells of the BBB – tanycytes for the BBB present within CVOs, and capillary endothelial cells that form the BBB in non-CVO locations – would be valuable. Additionally, the BCSFB at the choroid plexus (ChP) represents another potential site of brain entry. Though binding was not observed in the experiments conducted here, the ChP labelled with VT50-tagged liraglutide in a GLP-1R-independent manner (this was the only location with VT750-liraglutide signal in *Glp1r*^{-/-} mice) [179].

In vitro work examining whether GLP-1RAs can enter and subsequently be released from primary cells from these locations (i.e. ChP ependymal cells, tanycytes, and brain endothelial cells) would help interrogate the mechanistic basis for GLP-1RA uptake into the brain. Confocal video microscopy could be conducted to assess whether polarised uptake and extrusion of fluorescently-tagged agonist is seen [391]. Importantly, siRNA-mediated knockdown of the GLP-1R could be conducted to evidence that uptake is receptor independent. It should be noted that, of these three cell populations, only ependymal cells of the ChP have been formally reported to express the GLP-1R [459]. To better mimic the polarised orientation and existence of tight junctions between these cell populations at their physiological sites, cells could be grown upon transwell inserts and passage of agonist from apically- to basolaterally-located medium could be monitored [460]. Likewise, siRNA-mediated GLP-1R knockdown could be conducted in this paradigm. Studies such as these may confirm whether uptake

of exendin-4-based GLP-1RAs is indeed by transcellular diffusion in cells that form the BBB/BCSFB. Additionally, depending upon the tractability of said *in vitro* studies, investigation could extend to include non exendin-4-based GLP-1RAs including liraglutide, in order to investigate whether, as suggested [179], differences exist in the mechanisms of uptake.

Finally, it would be equally valuable to examine agonist uptake at cellular resolution after peripheral administration of the fluorescently-tagged agonists *in vivo*. Transgenic mice co-expressing a fluorescent reporter with the GLP-1R [91], [141] could be dosed with VT750-tagged agonist, and brain slices from regions of interest immunostained for neuronal, glial, epithelial or endothelial markers. This could allow determination of the cell subpopulations in which GLP-1RA is detected, and whether these express the GLP-1R.

4.4.6 Relevance of GLP-1RA-targeted brain regions

The hypothalamus and the DVC have well-established roles in GLP-1RA-mediated hypophagia [179], [306], [461]; agonist binding and activation of the GLP-1R in these regions may therefore have direct contributions to the anorectic effects of the biased GLP-1RAs. However, the consequences of agonist binding to GLP-1Rs in the SFO and OVLT are less clear. It is possible that these CVOs simply act as entry points and do not contribute to the anorectic effects of the biased GLP-1RAs. Supporting this, electrolytic lesion of the SFO in rats had no effect upon the anorectic effects of peripherally administered exendin-4 [113]. However, SFO lesioning did cause modest decreases in c-fos expression in the ARC and PVN in response to exendin-4 administration; thus, whilst not directly required for exendin-4's anorectic effects, it may modulate the activity of other regions that enact hypophagia [113]. Regarding the OVLT, no data appears to exist suggesting a direct role for this region in GLP-1R-mediated appetite suppression. However, both of the SFO and OVLT play important roles in osmoregulation [462], and may potentially contribute to GLP-1RA-mediated hypodipsia [463].

4.4.7 Brain uptake experiment caveats

A potential limitation of the brain and pancreata uptake experiments is the high dose of fluorescently-tagged agonist (100 nmol/kg) that was used. This dose was required for adequate detection of agonist at both 1-hour and 6-hour time points, and was recommended by the author of previous publications conducting similar studies [427]. It is possible that agonist distribution at this dose does not reflect that at lower doses used for physiological testing. Arguing against this, agonist-positive regions were matched (apart from the NTS) for mice administered 10 nmol/kg of exendin-4-VT750 (though admittedly this dose still produces maximal anorectic effects) (Figure 3.4). Moreover, the ARC, ME, NTS and AP all showed robust neuronal activation after low dose (0.6 nmol/kg) peripheral exendin-4 administration, suggesting the physiological relevancy of agonist-targeted regions [464].

The brain uptake experiments presented in this chapter report only the presence of agonist, which does not necessarily imply neuronal activation. It may be the case that subsequent patterns of neuronal activation differ for the biased GLP-1RAs, with relevant physiological impacts. It may therefore be pertinent to examine whether the pattern of brain activation of the biased GLP-1RAs differs. This could be done using traditional methods of examining c-fos expression in brain slices, or

in whole brains utilising an immunostaining compatible clearing protocol, as has been previously conducted [427], [464].

4.4.8 Summary

In summary, the results of this chapter evidence: a) a lack of correlation between the brain uptake and anorectic effects of the biased GLP-1RAs; b) the uptake of the biased GLP-1RAs into BBB-protected regions to be independent of GLP-1R endocytosis; and c) agonist access to the brain and pancreas to be equivalent. The exclusion of both the differential brain uptake and differential tissue access hypotheses strongly suggest that tissue bias is responsible for the divergent effects of the biased GLP-1RAs. An investigation into differential neuronal and β -cell responses to the biased GLP-1RAs is thus the subject of the following chapter.

5 Neuronal and β -cell Responses to Biased GLP-1RAs

5.1 Introduction

The anorectic and incretin effects of pharmacological GLP-1RAs are, at least in part, due to direct action upon GLP-1R-expressing CNS neurons and pancreatic β -cells, respectively [156], [163], [164], [180]. The results in Chapter 4 suggest that brain entry of exendin-4-based GLP-1RAs is independent of GLP-1R endocytosis, and that the divergence between the glucoregulatory and anorectic effects of biased GLP-1RAs after peripheral administration is not explained by differences in their ability to access central appetite regulating centres. Thus, an alternative possibility is raised wherein cell-specific factors influence the relative efficacy of biased agonists acting on neuronal *versus* pancreatic β -cells, thereby accounting for the observed divergence in their physiological effects.

In an attempt to characterise potential differences, neuronal responses to the agonists were evaluated *in vitro* and *ex vivo*. The existing literature suggests an important role for the arcuate nucleus of the hypothalamus in mediating the anorectic effects of long-acting GLP-1RAs [179], [210], [465]. Moreover, as evidenced in Chapter 4, all of the biased agonists readily accessed the ARC after peripheral administration. Thus, *in vitro* and *ex vivo* arcuate responses to the biased agonists were examined in this chapter. Whilst focussing upon this single anatomical location (*versus* all central GLP-1R-expressing nuclei) makes investigation manageable, it should be noted that GLP-1RA-induced hypophagia *in vivo* likely involves activation of multiple CNS regions [466].

Peptide concentration in the plasma and CSF taken from rats 1 hour after 100 nmol/kg peripheral administration were approximately 239 pM and 9.55 pM, respectively, as measured by ELISA (Figure 4.17; molecular weight of exendin-4 is 4187 g/mol). This suggests that peptide concentrations at CNS neurons mediating hypophagia are low in comparison to the supramaximal concentrations normally used *in vitro* to elicit robust cellular responses. Differences in agonist concentration may lead to differential receptor pharmacology in the two contexts, complicating clear comparisons. Direct administration of the biased GLP-1RAs into the CSF of the ventricular system would more closely mimic agonist concentrations *in vitro*, and for this reason, anorectic responses to the biased GLP-1RAs were assessed after intracerebroventricular (ICV) administration in this chapter.

5.1.1 Existing *in vitro* and *ex vivo* models of arcuate nucleus neurons

Given the difficult (but not impossible [467]) nature of studying at cellular resolution *in vivo*, *in vitro* and *ex vivo* models provide manageable systems for dissecting the cellular mechanisms underlying physiological phenomena. This approach also allows the effects of direct manipulation of the tissue of interest to be examined without influence from compensatory systemic responses. However, the isolated cellular responses in these systems are gross simplifications of *in vivo* physiology, and perhaps particularly so for neurons that form complex three-dimensional networks both within, and between specialised brain regions. Caution must therefore be applied when extrapolating *in vitro* results to *in vivo* physiology. A brief overview of the existing *in vitro* and *ex vivo* models for studying arcuate nucleus neurons is provided below, including some advantages and disadvantages of each approach.

5.1.1.1 Immortalised cell lines

The Belsham laboratory has generated over 50 different immortalised hypothalamic cell lines derived from adult and embryonic mouse tissue ("mHypo" cell lines; mHypoA for adult, and mHypoE or mHypoN for embryonic cell lines) [468]. Primary hypothalamic cells were immortalised by retroviral gene transfer of the oncogene Simian virus 40 large T antigen (SV40 T-Ag) and assessed for the expression of various hypothalamic neuropeptides and receptors, including NPY, POMC, the insulin receptor (IR), the ghrelin receptor (GHSR), and the GLP-1R [469]. Amongst others, mHypo cell lines with characteristics of gonadotrophin-releasing hormone (GnRH) [470], NPY/AgRP [471], and kisspeptin [472] ARC neuronal populations have been described and utilised for the *in vitro* study of their *in vivo* correlates. In addition to the mHypo cell lines, other laboratories have developed hypothalamic cell lines, including the GT1 cell lines (derived from GnRH-expressing neurons) that preceded mHypo cells [473], and alternative leptin-responsive [474] and kisspeptin neuron [475] cell lines.

The best-characterised population of ARC anorexigenic neurons are the POMC/CART neurons, and evidence suggests that they are relevant to the anorectic effects of pharmacological GLP-1RAs. Specifically, liraglutide bound to and was internalised by POMC/CART neurons after peripheral administration, and ablation of the ARC, or intra-ARC delivery of exendin(9-39) abolished liraglutide's anorectic effects [179]. More recently, it has been demonstrated that liraglutide both directly activates as well as increases the excitatory tone to primary POMC/CART neurons, and that leptin potentiated these effects [465]. Four mHypoA cell lines have been derived from POMC/CART neurons (mHypoA POMC/GFP 1-4); appropriately these neurons express POMC, CART, IR, LepR, and secrete α -MSH in response to ciliary neurotrophic factor (CNTF) or insulin [379]. However, only the mHypoA POMC/GFP3 cell line expresses the GLP-1R, which may reflect the fact that, *in vivo*, only 10% of ARC POMC neurons express the GLP-1R [83]. An additional POMC- and GLP-1R-expressing mHypoA cell line that has been demonstrated to respond to exendin-4 is the mHypoA 2/30 cell line [469], [476]. Thus, the mHypoA 2/30 and POMC/GFP3 cell lines are potential *in vitro* models of GLP-1R biology in anorexigenic ARC neurons.

The use of neuronal cell lines endows certain advantages. Their continual propagation facilitates high throughput experimentation and avoids poor yields of cells derived from primary tissue, thereby reducing animal usage. They are cost effective, and generate reproducible results due, in part, to their clonal nature overcoming cellular heterogeneity seen in primary culture of neurons. On the other hand, such a homogenous cellular population is also a drawback as *in vivo* neurons often receive innervation from multiple, phenotypically distinct neuronal populations, and additionally have their activity regulated by glial cells (e.g. astrocytes, microglia). Monoculture of neuronal cell lines omits these features. Moreover, three-dimensional neuronal connectivity is not captured by growth of neuronal cell lines in monolayers, and their continual propagation often results in loss of original neuronal characteristics (which are already an imperfect recapitulation of primary cells from the outset) [469].

5.1.1.2 Primary neuronal culture

Primary neurons better recapitulate the properties of neurons *in vivo* compared to immortalised cell lines, and thus are a superior model [477]. Well-established protocols exist for the isolation and study

of primary neurons [478], and adult mouse [479] and rat [480] hypothalamic neurons have been successfully isolated and studied. However, the non-proliferative nature and short *in vitro* life span of primary neurons means that obtaining sufficient quantities of cells can be challenging [477]. Primary cultures also contain a mixture of neurons and glia which, although more faithful to *in vivo* physiology, may mean that neuronal populations of interest represent a small fraction of the overall culture. The use of transgenic mouse lines with fluorescent reporters expressed under the control of specific neuropeptide or receptor promoters helps overcome this by allowing the isolation of those neurons of interest [477]. Relevant to the study of GLP-1R biology are the *Glp1r*-Cre/*Rosa26*-eYFP and *Glp1r*-Cre/*Rosa26*-tdRFP mouse lines [141], wherein the reporter is expressed only in cells in which Cre recombinase is produced under the control of the *Glp1r* promoter. These mouse lines would facilitate the isolation of GLP-1R-expressing neurons from primary cultures.

5.1.1.3 Stem cell-derived neurons

The study of human hypothalamic neurons has been hindered by the absence of human hypothalamic cell lines and the difficulty of collecting live cells from human hypothalami (given its relative inaccessibility) [481]. To overcome this, protocols have recently been developed for the production of hypothalamic neurons from human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) [481]–[484]. One of these protocols results in the production of neuropeptidergic neurons from several hypothalamic nuclei, including the ARC (POMC/ α -MSH and AgRP neuronal populations), PVN (oxytocin, arginine-vasopressin, and thyrotropin-releasing hormone neuronal populations), and LHA (orexin/hypocretin A and melanin-concentrating hormone neuronal populations) [483]. Two other protocols resulted in the production of predominantly ARC-like neurons characterised by POMC, CART, NPY, GHSR, IR, LepR and NPY receptor Y2 expression. Importantly, functional responses - increased p-AKT and p-STAT3, and the release of neuropeptides NPY and α -MSH – were demonstrated after treatment with leptin, insulin or ghrelin [481], [482].

Notably, the most recent of these reports generated iPSCs from super-obese (BMI ≥ 50) individuals, and demonstrated the preservation of common obesity-related SNPs and a dysregulated obesogenic transcriptomic profile in the ARC-like cells derived from these iPSCs [481]. Thus, the use of human ESCs and iPSCs facilitates the study of human hypothalamic-like neurons in both healthy and disease states. Though a powerful tool, stem cell derived hypothalamic neurons are still limited by their lack of three-dimensional cytoarchitecture, the absence of glia, and the occasional generation of neurons with mixed identity – for example, co-expression of POMC and NPY was observed in some ARC-like neurons [482].

5.1.1.4 Ex vivo brain slices

The substantial advantage of brain slice culture over other *in vitro* techniques is the preservation of brain cytoarchitecture, including the identity, relative abundance and connectivity of neurons and glia [485]. Brain slices can be used to study electrophysiology, connectivity, and cellular responsiveness to neurotransmitters/neuropeptides, and have been applied to the study of various hypothalamic nuclei, including the ARC [486]–[489], PVN [490], and LHA [491]. As with primary neuronal culture, the challenge of identifying specific neuronal populations can be surmounted by the use of transgenic mouse lines. Despite its notable advantages, *ex vivo* slice culture is not without its challenges: slice preparation is difficult and laborious; cell damage during slicing may result in further cell death by the

release of excitotoxic concentrations of calcium and glutamate; and *in vivo* connectivity is not perfectly recapitulated due to the severing of axon tracts between distant brain regions [485].

For the studies herein, mHypoA POMC/GFP3 cells were used for the majority of experiments given their tractability, and responses compared to those in mouse pancreatic β -cell line MIN6B1 in parallel assays, or as previously reported [323]. Additionally, Ca^{2+} responses were studied in *ex vivo* ARC brain slices to query *in vitro* findings in a more physiological model.

5.2 Aims

1. To examine arcuate nucleus neuron signalling responses to the biased GLP-RAs *in vitro* and *ex vivo*.
2. To assess the anorectic effects of biased GLP-1RAs after central administration.

5.3 Results

5.3.1 cAMP accumulation to biased GLP-1RAs in mHypoA POMC/GFP3 cells and MIN6B1 cells

The GLP-1R is G_s -coupled in both pancreatic β -cells and neurons; in the former, it is the predominant pathway linked to insulin secretion [297], [298], and in the latter, it is required for the anorectic effects mediated by specific neurocircuits in the NTS [306]. Given the demonstrated importance of G_s signalling in both cell types, cAMP accumulation responses were assessed in mHypoA POMC/GFP3 cells and mHypoA 2/30 cells. Exendin-4 induced robust responses in mHypoA POMC/GFP3 cells that could be completely antagonised by exendin(9-39), thereby evidencing their dependency upon the GLP-1R (Figure 5.1 A). By contrast, cAMP responses were undetectable in mHypoA 2/30 cells (data not shown), and thus all subsequent assays were performed in mHypoA POMC/GFP3 cells.

Acute cAMP responses (5 minute stimulation) in mHypoA POMC/GFP3 cells revealed high signalling efficacy and matched potency for exendin-4 and exendin-asp3, but reduced efficacy and potency for exendin-phe1 (Figure 5.1 B, Table 5.1). These results differed from cAMP responses in mouse pancreatic β -cell line MIN6B1, where exendin-phe1 exhibited reduced potency, but still acted as a full agonist (Figure 5.1 D, Table 5.1) [323]. The difference in exendin-phe1-induced cAMP responses can, in principle, be explained by differences in the level of GLP-1R expression and the resulting impact on amplification of intracellular signalling with agonists of different inherent efficacies. RNAseq data (see below; Figure 5.2 E) revealed GLP-1R expression to be over 2000-fold lower in mHypoA POMC/GFP3 cells as compared to MIN6B1 β -cells. Admittedly, it is accepted that de-differentiation of immortalised cell lines may mean that the expression of non-essential proteins is artificially reduced compared to in primary tissues. However, it is notable that GLP-1R mRNA transcripts were >100 times less abundant in GLP-1R-expressing vagal afferent neurons than in pancreatic islets [141], and also considerably lower in mouse hypothalamic and brainstem *versus* pancreatic islet tissue [492]. These data indicate that neuronal GLP-1R expression levels may indeed be considerably lower than in β -cells.

Agonists with inherently low efficacy for G_{α_s} recruitment rely on sufficient receptor density to maximally activate adenylate cyclase and generate a full cAMP response; under these circumstances signal amplification means they can behave as full agonists for cAMP. In contrast, when receptor density is reduced, even maximal receptor occupancy may fail to translate to a full downstream response, meaning the ligand acts as a partial agonist. A high efficacy agonist, on the other hand, can generate a full response even at submaximal receptor occupancy – the concept of “spare receptors”. Of note, ongoing work in the department confirms exendin-phe1 is indeed a low efficacy agonist for G_{α_s} recruitment (data not shown). In summary, the interplay between agonist efficacy and spare receptors has a greater impact upon weak agonist exendin-phe1, causing it to act acutely as a partial agonist for cAMP in mHypoA POMC/GFP3 cells but a full agonist in MIN6B1 cells, with a caveat that the difference here may be artificially magnified if GLP-1R expression in the neuronal cell line is even lower than in primary cells.

Moreover, and importantly, comparison of exendin-4 EC_{50} values in both cell types revealed that signalling potency was approximately 100-fold lower in neurons than in beta cells (Table 5.1). This observation is also compatible with differences in receptor density discussed above, as the law of

mass action ensures that, assuming exendin-4 has the same K_d in both cell types, the same ligand concentration will mean the fraction of occupied surface receptors will be the same in each case. However, when receptor expression is high, a lower fractional occupancy is required to saturate the available adenylate cyclase pool. This observation is also notable in view of the dose relationship of acute anti-hyperglycaemic *versus* anorectic effects observed in Chapter 3, which showed that significantly higher doses of GLP-1R agonist were required to suppress food intake than to reduce blood glucose (Figure 3.4, Table 3.1). This difference is worth highlighting to aid the interpretation of a number of experiments performed in this chapter and from previous chapters, because:

- It is reasonable to speculate that GLP-1R desensitisation mechanisms in neurons also might require somewhat higher agonist doses than in β -cells and these may not be achieved via reasonable pharmacological dose scales *in vivo*; and
- Many of the pharmacological tests described below were performed to probe desensitisation-related differences at an agonist dose selected to ensure supramaximal stimulation of both cell types.

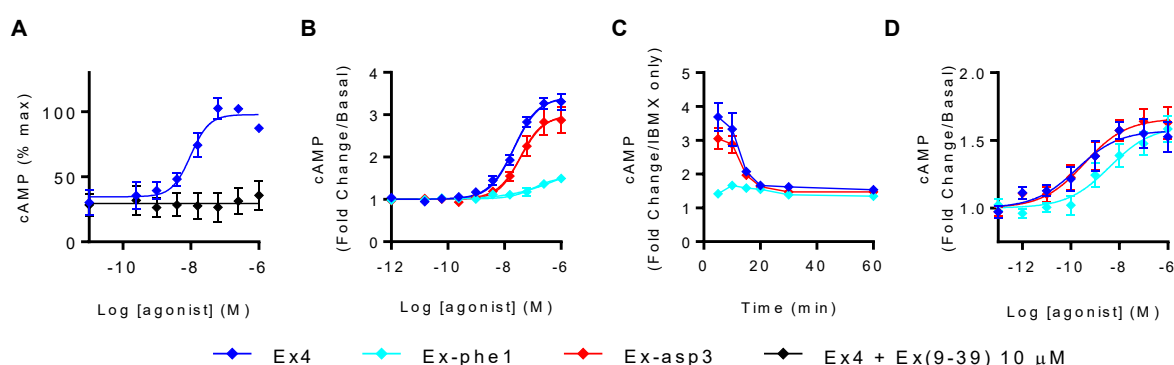


Figure 5.1. cAMP accumulation responses in mHypoA POMC/GFP3 cells and MIN6B1 cells.

(A) cAMP responses in mHypoA POMC/GFP3 cells to exendin-4 with or without exendin(9-39) at 10 μ M; 5 minute stimulation with 100 μ M IBMX, $n=3$. Data are expressed as a percentage of the maximal response in the assay per treatment with a four-parameter logistic fit, and are presented as mean $\pm$ SEM. **(B)** cAMP responses in mHypoA POMC/GFP3 cells after 5 minutes of agonist stimulation in the presence of 500 μ M IBMX, $n=5$. Data are expressed relative to IBMX-only response with a four-parameter logistic fit, and are presented as mean $\pm$ SEM. **(C)** Time course of cAMP accumulation in mHypoA POMC/GFP3 cells after treatment with agonist at 200 nM in the presence of 500 μ M IBMX, $n=6$. Data are presented as mean $\pm$ SEM after normalisation to IBMX-only response for the corresponding time point. **(D)** As in (A) but in MIN6B1 cells, $n=6$.

	mHypo POMC/GFP3				
Peptide	pEC ₅₀	Δ logEC ₅₀	EC ₅₀ Ratio	E _{max} (fold change)	Ratio
Ex4	7.65 $\pm$ 0.08			3.30 $\pm$ 0.137	
Ex-phe1	7.18 $\pm$ 0.106	0.58 $\pm$ 0.05	4.12	1.61 $\pm$ 0.123	0.49 $\pm$ 0.02
Ex-asp3	7.37 $\pm$ 0.196	0.08 $\pm$ 0.04	1.33	3.18 $\pm$ 0.306	0.96 $\pm$ 0.02
	MIN6B1				
Ex4	9.61 $\pm$ 0.11			1.57 $\pm$ 0.03	
Ex-phe1	8.63 $\pm$ 0.19	0.98 $\pm$ 0.18	9.47	1.63 $\pm$ 0.04	1.02 $\pm$ 0.01
Ex-asp3	9.44 $\pm$ 0.09	0.18 $\pm$ 0.13	1.49	1.65 $\pm$ 0.04	0.97 $\pm$ 0.00

Table 5.1. Potency and efficacy of biased GLP-1RAs for cAMP accumulation in mHypoA POMC/GFP3 cells and MIN6B1 cells.

Values derived from data in Figure 5.1. “ $\Delta\log EC_{50}$ ” is the difference in $\log EC_{50}$ values between exendin-4 and the denoted peptide. “ EC_{50} Ratio” and “Ratio” describes the peptide’s EC_{50} value and E_{max} value, respectively, relative to that of exendin-4. Data reported as mean $\pm$ SEM from 6 independent experiments.

In keeping with the reduced receptor density being a limiting factor for GLP-1R signalling in mHypoA POMC/GFP3 cells, time course experiments revealed cAMP signalling responses to all agonists were transient, with cumulative cAMP accumulation after 20 minutes of agonist exposure being indistinguishable from non-agonist treated cells after an initial peak, suggesting rapid desensitisation (Figure 5.1 C). Moreover, prolonged incubation periods (16 hours) yielded a complete lack of response to the agonists (data not shown). This is in direct contrast to β -cell lines where cAMP responses to slow-internalising exendin-phe1, albeit small, were reliably detected after 24-hour incubation [323]. The transient responses observed in mHypoA POMC/GFP3 cells may reflect the need for high fidelity signalling in POMC neurons that, in their *in vivo* environment, integrate signals from heterogeneous populations of synapsing neurons. The need to discriminate individual inputs would necessitate rapid response termination. Thus, cAMP accumulation assays in mHypoA POMC/GFP3 cells suggest the existence of tissue specific signalling differences that affect responses to all agonists (temporal aspects), as well as those that cause agonist-specific changes (reduced signalling efficacy for weak agonists due to low GLP-1R expression).

5.3.2 Transcriptomic analysis of the cellular response to biased GLP-1RAs in mHypoA POMC/GFP3 and MIN6B1 cells.

Whilst native GLP-1 is rapidly degraded and provides only a transient stimulus, prolonged agonist exposure *in vitro* more closely mimics the continual exposure of tissues *in vivo* to pharmacological GLP-1RAs. This is of heightened relevance in light of the drive to develop compounds with weekly dosing frequencies such as semaglutide and dulaglutide. However, the lack of detectable cAMP accumulation in mHypoA POMC/GFP3 cells after 16-hour incubations precluded this as a readout of the effects of sustained agonist exposure. Measurement of the release of the anorexogenic neuropeptide α -MSH, a cleavage product of POMC, was attempted as an approach analogous to measurement of insulin secretion in β -cells, but could not be detected by ELISA (data not shown). Of note, only a minor increase in α -MSH release was detected using this cell line with KCl stimulation to maximally stimulate granule exocytosis [379], suggesting that despite its POMC lineage, core downstream functionality is lacking. Nevertheless, as acute GLP-1R cAMP signalling responses could clearly be detected, an alternative readout of prolonged signalling was sought. Here, RNAseq was used to study agonist-induced transcriptomic changes in mHypoA POMC/GFP3 cells after 16-hour incubation at a single high agonist dose. Library preparation and DESeq2 analysis was kindly performed by Grazia Pizza and Dr Aida Martinez Sanchez in the Section of Cell Biology, Imperial College London. Responses were expressed relative to gene expression in vehicle-treated cells and are summarised in Figure 5.2. For comparison, an RNAseq dataset from MIN6B1 cells similarly treated with biased GLP-1RAs at 100 nM for 16-hours, was provided by a collaborator, Dr Alejandra Tomas, Imperial College London, and subjected to a limited analysis (Figure 5.2 F).

Unlike prolonged cAMP assays, differential responses to the agonists in mHypoA POMC/GFP3 cells were detected at the transcriptional level. Exendin-4 caused significant changes in the expression of fifty genes; by contrast, exendin-phe1 and exendin-asp3 only significantly affected the expression of

three and two genes, respectively. In contrast, in MIN6B1 cells (Figure 5.2 F), the relative relationship between exendin-4 and exendin-phe1 was reversed; exendin-4 treatment caused significant changes to the expression of 4 genes, whilst exendin-phe1 significantly affected the expression of 107 genes. Exendin-asp3 was similarly ineffective in inducing long-term transcriptional change, significantly affecting the expression of only 5 genes. Thus, unlike for previously documented effects on insulin secretion, and now for transcriptional response patterns in pancreatic β -cells, exendin-phe1 did not exhibit prolonged signalling effects in neurons relative to exendin-4, which is likely to be linked to its reduced acute signalling amplitude (Figure 5.1 B, Table 5.1). By contrast, responses to exendin-asp3 mirrored those in β -cells, with diminished responses after prolonged exposure, despite high signalling efficacy acutely. This time-dependent diminution of exendin-asp3's effects is likely to be linked to its enhanced β -arrestin recruitment and/or tendency to prevent GLP-1R recycling [323].

Additionally, expression levels of GLP-1R, β -arrestin-2, and $G\alpha_s$ in mHypoA POMC/GFP3 cells were evaluated, and compared to results from MIN6B1 cells (Figure 5.2 E). Results were normalised to the average expression of endogenous control genes *Gapb1*, *D8Ertd738e*, and *Psmb2* given recent transcriptomic analysis evidencing their uniform expression across 17 mouse tissues, and thus their improved suitability to commonly used housekeeping genes, the expression of which differed notably between tissue types [493]. Indeed, between mHypoA POMC/GFP3 and MIN6B1 cells, the expression of commonly used housekeeping genes *Actb* (β -actin), *Gapdh* (Glyceraldehyde 3-phosphate dehydrogenase), and *Rsp18* (18s rRNA) was highly variable. As aforementioned, expression of *Glp1r* in mHypoA POMC/GFP3 cells was over 2000-fold lower than housekeeping genes, and *Glp1r* expression in MIN6B1 cells. Expression of *Gnas* ($G\alpha_s$) was matched between cell lines, but *Arrb2* (β -arrestin-2) expression in MIN6B1 cells was two-fold of that in mHypoA POMC/GFP3 cells.

Examining the identity of differentially expressed genes in mHypoA POMC/GFP3 cells, there was no overlap in the genes affected by the different agonists. Many of the genes affected by exendin-4 were not overtly linked to physiological effects mediated by POMC neurons, for example those involved in regulation of the extracellular matrix (*Marcks*, *Matn2*), or axon guidance and cell migration (*Plxdc1*, *Edil3*, *Cdh13*, *Has2*). Broadly speaking, genes for proteins with neuroprotective roles were upregulated, whilst those implicated in neurodegenerative disease exacerbation and pro-inflammatory effects were downregulated. Genes of interest and their relevant effects in neurons are summarised in Table 5.2. Of particular interest are humanin and thioredoxin-interacting protein given their dual effects in neurons and pancreatic β -cells, and ataxin 2 and osteoglycin for their metabolically related effects.

The overall pattern of exendin-4-induced gene expression changes is consistent with the known neuroprotective and neurotrophic effects of GLP-1RAs [424]. Indeed, clinical trials are currently underway to assess the efficacy of liraglutide in the treatment Alzheimer's disease (AD; clinical trial identifier NCT01843075) and Parkinson's disease (PD; clinical trial identifier NCT02953665), and a small phase II trial suggested exenatide to alleviate symptoms of Parkinson's disease [494]. Moreover, the recognition of neuronal dysfunction as a feature of obesity and T2DM increases the attractiveness of GLP-1RAs as treatments for these metabolic disorders [495]. Specific components of the biased GLP-1RA-mediated MIN6B1 transcriptional responses are not disclosed in detail here as these are part of a separate piece of work.

Gene	Protein	Neuronal role/effect		Description	Refs
Metabolism-related					
<i>Atxn2</i>	Ataxin 2	Anorectic	↑	Polyglutamine protein with an important role in RNA metabolism and translation regulation; mutations in <i>Atxn2</i> best known for their aetiology in spinocerebellar ataxia type 2. SNPs in <i>Atxn2</i> are associated with metabolic disorders, and <i>Atxn2</i> knockout/depletion has been demonstrated to cause hyperphagia, obesity, altered lipid metabolism, and insulin resistance in animal models.	[496] – [499]
<i>Ogn</i>	Osteoglycin	Orexigenic	↓	Secreted proteoglycan that coordinates bone formation with overall energy balance; induces hyperphagia by enhancing NPY expression in the hypothalamus, and improves glucose uptake by modulating insulin secretion and sensitivity.	[500]
Neurodegeneration/neuroprotection					
<i>mt-Rnr2</i>	Humanin	Neuroprotective	↑	Peptide encoded in mitochondrial genome with neuroprotective effects in <i>in vitro</i> and preclinical models of Alzheimer's disease; also has cytoprotective effects in pancreatic β -cells, and potentiates insulin secretion <i>in vivo</i> .	[501] – [504]
<i>Sgk1</i>	Serum/glucocorticoid regulated kinase 1	Neuroprotective	↑	Serine-threonine kinase with cytoprotective effects in various cell types including neurons; appears to play a neuroprotective role in preclinical models of neurodegenerative diseases.	[505] – [507]
<i>Timp1</i>	Tissue inhibitor of metalloproteinase 1		↑	Regulator of metalloproteinase activity; has been shown to have anti-apoptotic effects in neurons.	[508], [509]
<i>Ereg</i>	Epiregulin	Neuroprotective	↑	Member of the epidermal growth factor family of peptide hormones; demonstrated to inhibit ER stress and subsequent neuronal death.	[510]
<i>Txnip</i>	Thioredoxin interacting protein	Pro-apoptotic	↓	Demonstrated to be pro-apoptotic in haemorrhagic brain injury and PD; mediates glucotoxicity and apoptosis in pancreatic β -cells.	[511], [512]
<i>ApoE</i>	Apolipoprotein E	Pro-AD development	↓	Principal cholesterol carrier in the brain; strongly implicated in the development of AD by modulating amyloid β aggregation and deposition.	[513]
<i>ApoD</i>	Apolipoprotein D	Pro-neurological disorders (putative)	↓	Secreted glycoprotein and lipid carrier with no homology to other apolipoproteins; purported to play a role in neurological disorders and nerve injury.	[514]
<i>Enpp2</i>	Autotaxin	Pro-AD	↓	Secreted enzyme that hydrolyses extracellular lysophospholipids into lipid mediator lysophosphatidic acid; recently implicated in AD pathogenesis.	[515]
Inflammation					
<i>Lbp</i>	Lipopolysaccharide binding protein	Pro-inflammatory (putative)	↓	Acute phase protein that binds to, and enhances the responsiveness of immune cells to LPS. LPS itself is implicated in neurodegenerative decline.	[516], [517]

Table 5.2. Differentially expressed genes of interest after exendin-4 treatment in mHypoA POMC/GFP3 cells.

Genes of interest and their role in neurons from the 50 genes that showed differential expression after exendin-4 treatment in mHypoA POMC/GFP3 cells. Upwards arrow denotes upregulation, and downwards arrow denotes downregulation after exendin-4 treatment.

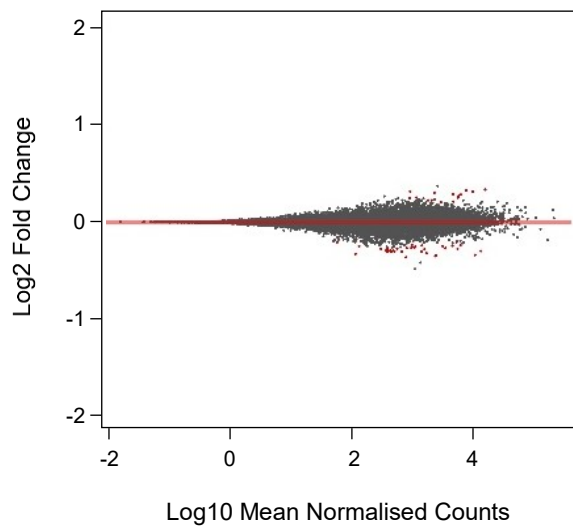
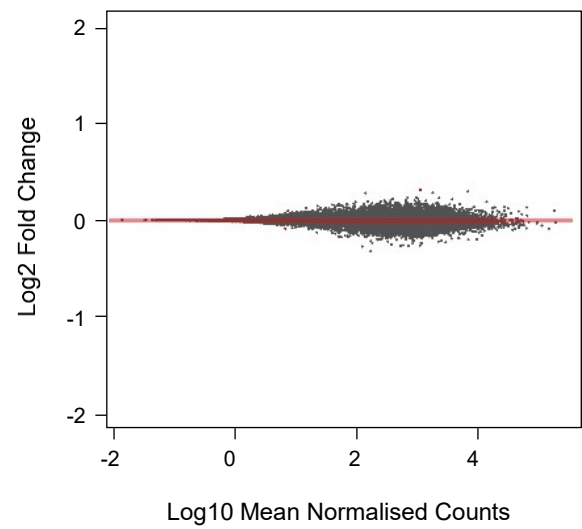
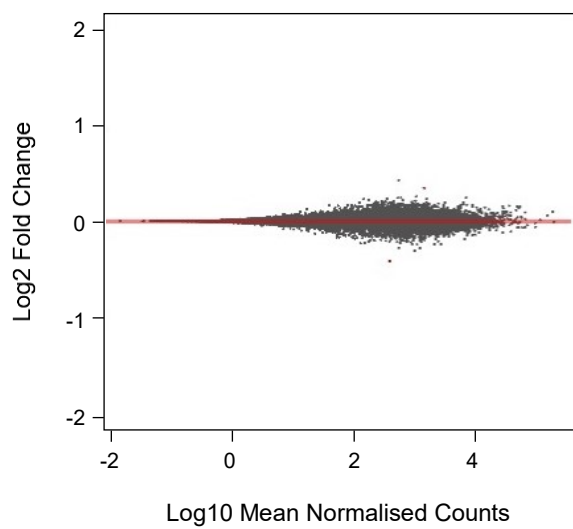
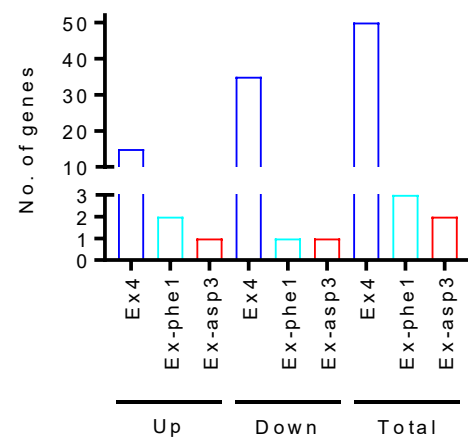
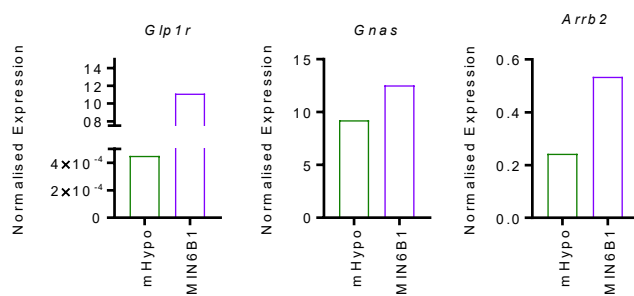
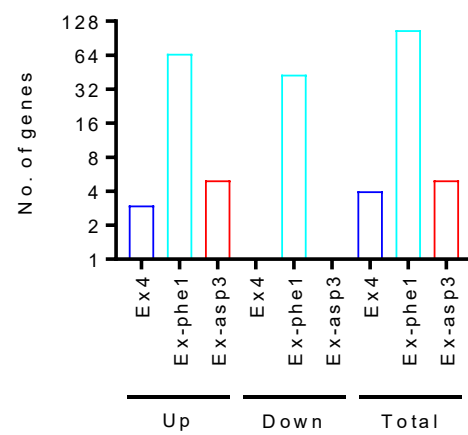
A**B****C****Vehicle vs. Ex-asp3****D****mHypoA POMC/GFP3****E****F****MIN6B1**

Figure 5.2. Transcriptomic changes in mHypoA POMC/GFP3 and MIN6B1 cells after stimulation with biased GLP-1RAs.

(A) MA plots indicating the log2 fold change in gene expression relative to vehicle-treated cells after 100 nM, 16-hour exendin-4 treatment in mHypoA POMC/GFP3 cells, n=3 independent experiments. Individual points represent a single gene. Relative expression values are plotted against the average number of counts for the gene across experiments. Positive and negative values denote gene upregulation and downregulation, respectively. Red dots indicate genes with expression significantly different from vehicle. Wald test with Benjamini-Hochberg adjustment to correct for multiple comparisons, significance achieved where adjusted p value <0.1. (B) As in (A) but after exendin-phe1 treatment. (C) As in (A) but with exendin-asp3 treatment. (D) Number of genes significantly upregulated or downregulated relative to vehicle-treated cells, as determined from data in (A)-(C). (E) Expression of *Glp1r*, *Gnas* and *Arrb2* in mHypoA POMC/GFP3 and MIN6B1 cells, as determined by RNAseq. Gene counts were separately normalised to those of *Gapb1*, *D8Ertd738e*, and *Psmb2*, and the average of the three normalised values reported. (F) Number of genes significantly upregulated or downregulated in MIN6B1 cells after 100 nM, 16-hour treatment with exendin-4, exendin-phe1 or exendin-asp3, n=3 independent experiments. Significant differences were determined relative to expression in vehicle-treated cells. Wald test with Benjamini-Hochberg adjustment to correct for multiple comparisons, significance achieved where adjusted p value <0.1.

To summarise how the *in vitro* cAMP and transcriptomic responses measured in mHypoA POMC/GFP3 cells correlate with the appetite suppressive effects of each agonist *in vivo*, see Table 5.3. The cell model recapitulates to a certain extent the anorectic characteristics of the different agonists. Exendin-4 shows high *in vitro* signalling efficacy at short- and long-term time points reflecting its sustained anorectic effects *in vivo*. With exendin-phe1, acute responses were lower in the cell model (Figure 5.1 B) than might be expected from the only subtly reduced acute anorectic effect *in vivo* (Figure 3.3 A-D), which may be due to even lower levels of GLP-1R expression in mHypoA POMC/GFP3 cells compared to the already low levels in primary anorectic neurons. Crucially though, exendin-phe1's reduced efficacy at both acute and sub-chronic time points in neurons relative to exendin-4 may be relevant to why it fails to induce stronger anorectic effects, despite its prolonged signalling in β -cells. Finally, exendin-asp3 displays pronounced effects acutely in both systems which wane with time *in vitro* (Figure 5.1 B and Figure 5.2 D) and to a much lesser extent, *in vivo* (Figure 3.3 and Figure 3.4 F). The higher and more stable concentrations of agonist in the closed *in vitro* stimulation system may magnify high dose-related receptor desensitisation, and thus exendin-asp3's enhanced tendency to drive GLP-1R desensitisation, which is not nearly as extensive in the *in vivo* setting as the achieved ligand concentrations are not as high.

	Acute effect		Sub-chronic effect	
	mHypoA cAMP	<i>In vivo</i> hypophagia	mHypo RNAseq	<i>In vivo</i> hypophagia
Ex4	High	High	High	High
Ex-phe1	Low	Slightly reduced	Low	Slightly reduced
Ex-asp3	High	High	Low	Slightly reduced

Table 5.3. Comparison of the *in vitro* and *in vivo* effects of the biased GLP-1RAs in neurons.

A summary of the effects of the biased GLP-1RAs *in vitro* and *in vivo* in acute and sub-chronic settings. "Acute effect" and "sub-chronic effect" in mHypoA POMC/GFP3 cells refer to 5-minute cAMP accumulation responses and 16-hour transcriptional responses, respectively. "Acute effect" and "subchronic effect" *in vivo* refer to the magnitude of hypophagia at 1 hour and 8 hours, respectively.

5.3.3 GLP-1R desensitisation in mHypoA POMC/GFP3 cells

Whilst the β -cell-specific benefits of exendin-phe1 were not observed in mHypo POMC/GFP3 cells, sustained exposure to β -arrestin-biased exendin-asp3 resulted in reduced transcriptional change relative to exendin-4, suggesting that augmented desensitisation and reduced recycling rate for exendin-asp3 may also be a feature of neuronal signalling, at least *in vitro*. Moreover, the kinetics of the cAMP responses in Figure 5.1 C suggests rapid onset of desensitisation. cAMP responses to re-challenge with exendin-4 were therefore assessed after fixed, high-dose agonist pre-treatment, washout, and a 1-hour recovery period for receptor resensitisation. Prior exposure to all agonists blunted subsequent responses to exendin-4, although, consistent with pancreatic β -cells, response diminution was somewhat less for G-protein-biased exendin-phe1 (Figure 5.3 A, B). Under these experimental conditions, exendin-4 and exendin-asp3 pre-treatment resulted in similar responses to re-challenge. This suggests that additional factors contribute to exendin-asp3's reduced effects upon transcriptional responses in neurons, or that longer time periods may be required to observe exendin-asp3's signalling deficit relative to exendin-4. In particular, the reduced recycling rate of GLP-1R after exposure to exendin-asp3 might take longer to manifest than the one hour resensitisation period used here. Changes in potency for the exendin-4 re-challenge were not observed with any of the agonist pre-treatments (Figure 5.3 C).

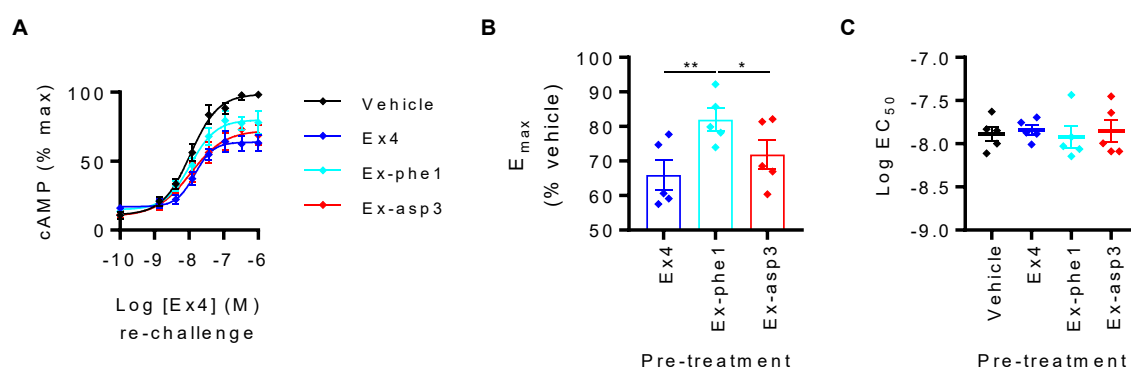


Figure 5.3. GLP-1R desensitisation in mHypoA POMC/GFP3 cells.

(A) cAMP responses in mHypoA POMC/GFP3 cells pre-treated for 10 minutes with 100 nM exendin-4, exendin-phe1, or exendin-asp3 to induce desensitisation, followed by one hour recovery and then re-challenge for with exendin-4 at the indicated concentrations. 5 minute exendin-4 re-challenge in the presence of 500 μ M IBMX, $n=5$. Data are expressed as a percentage of the maximal response per treatment with a four-parameter logistic fit, and are presented as mean $\pm$ SEM. **(B)** E_{max} values as derived from data in (A). One-way randomised block ANOVA with Tukey's test. * $p<0.05$, ** $p<0.01$. **(C)** Log EC_{50} values as derived from data in (A). Data are presented as mean $\pm$ SEM.

5.3.4 GLP-1R internalisation in mHypoA POMC/GFP3 cells

The reduced receptor desensitisation observed with exendin-phe1 pre-treatment may be due to its beneficial trafficking profile (reduced GLP-1R internalisation and increased GLP-1R recycling) seen in pancreatic β -cells, resulting in a larger fraction of sensitised receptors at the plasma membrane capable of responding to subsequent agonist challenge. Agonist-induced GLP-1R internalisation was therefore assessed in mHypoA POMC/GFP3 cells. The low level of receptor expression prevented detection of either FITC- or VT750-tagged agonists (data not shown), and thus cells were transiently

transfected with C-terminus EGFP-tagged GLP-1R. GFP fluorescence was evenly present throughout the cytoplasm and at the plasma membrane under vehicle conditions. However, after 30 minutes of stimulation, extensive cytoplasmic puncta were observed in exendin-4- and exendin-asp3-treated cells indicating trafficking of GLP-1R to endosomes, but not in exendin-phe1-treated cells (Figure 5.4). A similar pattern of internalisation was seen for the agonists in pancreatic β -cells [323] and HEK293 cells (e.g. Figure 4.11), suggesting that exendin-phe1 may maintain its characteristic trafficking profile across cell types, which may be responsible for the reduced desensitisation seen with this agonist.

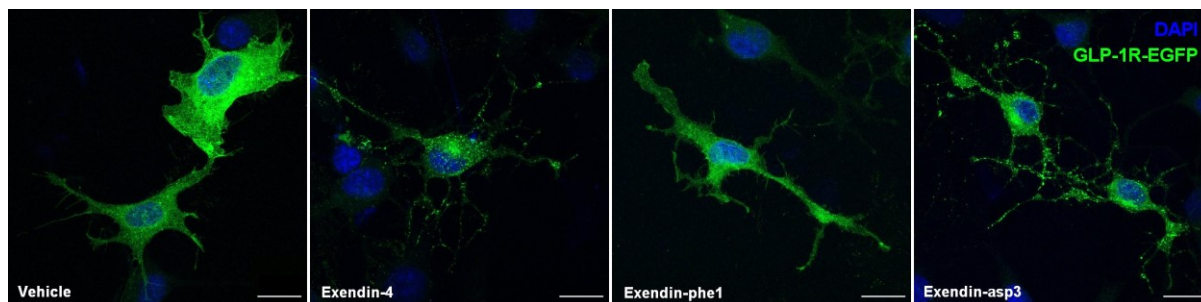


Figure 5.4. GLP-1R internalisation in mHypoA POMC/GFP3 cells.

Representative maximum intensity projection confocal images indicating GLP-1R internalisation after 30 minutes of agonist stimulation at 100 nM in mHypoA POMC/GFP3 cells transiently transfected with N-terminus EGFP-tagged GLP-1R. $n=3$ experiments, scale bar denotes 20 μm .

5.3.5 Ca^{2+} responses in *ex vivo* brain slices from *Glp1r*-Cre/GCaMP3 mice

Noting the limitations of the mHypo POMC/GFP3 cell line highlighted above as a model for GLP-1R-expressing neurons, responses to GLP-1R agonist stimulation were further evaluated in *ex vivo* brain slices, with the advantage that this system better recapitulates the complexity of arcuate nucleus connectivity *in vivo*. Brain slices were taken from mice expressing the genetically encoded Ca^{2+} reporter GCaMP3 [518] in which the *Glp1r* promoter drives expression of Cre recombinase to excise a loxP-flanked stop codon inserted prior to the GCaMP3 fusion gene (GLP-1R-Cre-GCaMP3 mice). Thus, GCaMP3 is expressed only in cells with an active *Glp1r* promoter (or, which is a caveat for this class of transgenic mouse, if *Glp1r* was expressed during their lineage development). GCaMP3 fluorescence intensity increases in response to intracellular Ca^{2+} accumulation, acting as a surrogate marker for neuronal depolarisation [519]. It should be noted that various GCaMPs, although commonly used in neurobiology, can in themselves exert deleterious effects on neuronal function [520], which a recent GCaMP refinement has sought to address [521]. Bearing these caveats in mind, GLP-1R-Cre-GCaMP3 mice were used to assess agonist-induced activity in GLP-1R-expressing neurons of the ARC. *Glp1r*-Cre/GCaMP3 mice were kindly provided by Professor Stefan Trapp from University College London; experiments were conducted in the Trapp laboratory by Wanqing Jiang with the assistance of the author. Given the labour-intensive nature of the experiments, responses to only oppositely biased exendin-phe1 and exendin-asp3 were examined.

Results are summarised in Figure 5.5. Ca^{2+} responses were examined in ARC-containing hypothalamic slices in response to acute stimulation with exendin-phe1 and exendin-asp3 (Figure 5.5 A), before agonist washout and a 2-hour period for receptor resensitisation. Slices were then re-challenged with exendin-4 to assess GLP-1R desensitisation (Figure 5.5 B), as in mHypoA

POMC/GFP3 cells. A longer recovery period was deployed relative to *in vitro* desensitisation experiments due to the practicalities of the experiment. Representative traces of acute stimulation and re-challenge reveal a small magnitude of average response to agonist at both time points, in comparison to the large increases in fluorescence seen in response to supraphysiological levels of glutamate. This suggests GLP-1R stimulation, at least in this experimental setting, engages only a portion of the overall calcitropic effector system in these neurons, and may therefore only modulate neuronal excitability rather than directly causing neuronal activation.

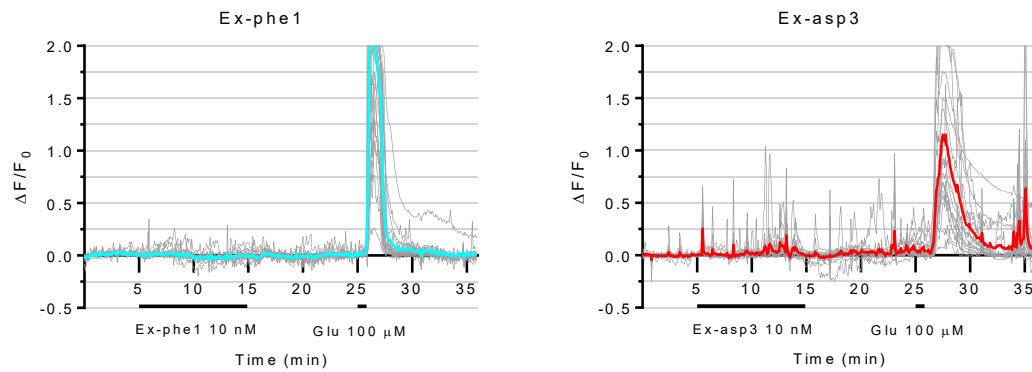
Quantification of responses by taking the area under the curve of population averages (Figure 5.5 C) or of all individual neurons (Figure 5.5 D, E) revealed relative agonist responses consistent to those seen in mHypoA POMC/GFP3 cells. Acutely, exendin-asp3 increased neuronal activity whereas exendin-phe1 had a minor effect. By contrast, exendin-phe1 pre-treated slices exhibited larger responses to re-challenge with exendin-4 in comparison to exendin-asp3. Differences in re-challenge responses were not statistically significant, likely due to a lack of power given the small magnitude of neuronal responses in these experiments. Ideally, further repeats would have been conducted to confirm the observed trend, but this was not possible due to time constraints.

Thus, in both mHypoA POMC/GFP3 cells *in vitro* and in GLP-1R-expressing ARC neurons *ex vivo*, β -arrestin-biased exendin-asp3 appears to cause greater GLP-1R desensitisation than G-protein-biased exendin-phe1. This relationship mirrors that in pancreatic β -cells [323]. However, the anorectic responses to the two agonists are approximately matched after peripheral administration, with at most a minor loss in efficacy with exendin-asp3 with longer exposures, suggesting that the augmented desensitisation induced by exendin-asp3 *in vitro* and *ex vivo* is not manifested to the same extent in neurons *in vivo*. Exendin-asp3's signalling properties in neurons thus appear to be influenced by the concentrations to which neurons are exposed, with only supramaximal, stable agonist concentrations *in vitro/ex vivo* being sufficient to drive maximal GLP-1R desensitisation. Indeed, measured agonist concentrations in the CSF and plasma from rats were 9.55 and 239 pM, respectively (Figure 4.17), 500-fold to 10 000-fold lower than the EC₅₀ of exendin-4 in mHypoA POMC/GFP3 cells (Table 5.1). Caveats for these numerical comparisons are that samples may not have been taken at the true pharmacokinetic peak (note for example that another study reports exendin-4 concentrations can reach >10 nM in mice with adequate dosing [445]), higher concentrations might be achieved in mice due to faster absorption kinetics, and that spatially constrained signalling events may still occur within cellular nanodomains without inducing increases in total cellular cAMP (for example) large enough to be detected using biochemical assays.

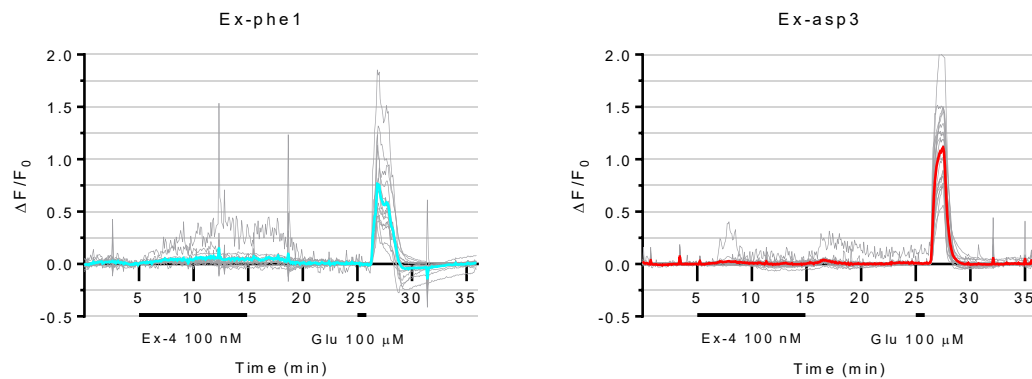
At first, this may appear to conflict with what occurs in pancreatic β -cells *in vivo*, where exendin-asp3 experiences a time-dependent loss of efficacy relative to exendin-phe1 in GTTs, despite the pancreas being exposed to equivalent agonist concentrations to the brain (Figure 4.18). However, agonist concentrations required for β -cell responses were *100-fold lower* than those for equivalent neuronal responses *in vitro* (Table 5.1), presumably attributable to high levels of GLP-1R expression (Figure 5.2) and signal amplification in the G α_s /adenylate cyclase/cAMP pathway in the former, but not latter cell type. Due to high β -cell responsivity, profound acute anti-hyperglycaemic effects can be elicited even with relatively low pharmacological doses after peripheral administration. As it can be plausibly suggested that the threshold for initiation of desensitisation mechanisms in β -cells is also lower than in neurons, it follows that only in exendin-phe1-exposed β -cells do sufficient levels of sensitised GLP-1R remain at the plasma membrane (due to its favourable trafficking profile) for anti-hyperglycaemic

effects to be manifested in the sub-chronic setting (delayed glucose challenge) when agonist dose is relatively low. Indeed, if exendin-asp3 is administered at high enough doses it is capable of robust anti-hyperglycaemic effects in delayed GTTs (Figure 3.4). This can be explained as follows: although fewer sensitised receptors are present at the cell membrane relative to exendin-phe1 at this late time point, the supramaximal dose likely allows sufficient receptor occupancy to facilitate a maximal cellular response in β -cells when combined with high signal amplification in the $G\alpha_s$ pathway. Restating these concepts as applied to anorectic neurons, the reduced sensitivity of the latter system, as indicated by the 100-fold reduction in potency to exendin-4 for cAMP production, is likely also to apply to the desensitisation mechanisms which govern the influence of signal bias on longer term agonist efficacy. Consequently, at the moderate or even “reasonably high” agonist doses typically used *in vivo*, desensitisation processes are not fully activated. The failure to observe the expected rank order of agonist efficacy for food intake at doses where clear differences are seen for glucose regulation could therefore be attributable to the relative sensitivities of the two systems (see Figure 5.7 for a schematic representation of these concepts).

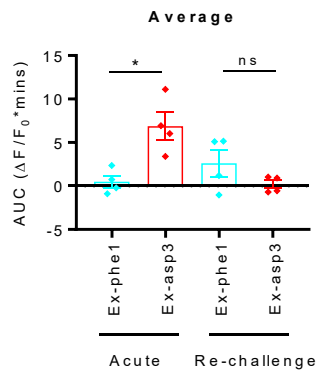
A Acute Stimulation



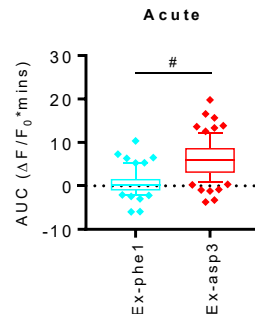
B Re-challenge



C



D



E

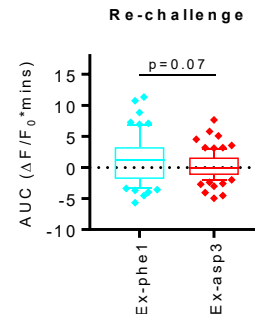


Figure 5.5. Ca^{2+} responses in ARC neurons in *ex vivo* brain slices from Glp1r-Cre/GCaMP3 mice.

(A) A representative trace of intracellular Ca^{2+} responses in ARC neurons in *ex vivo* slices from Glp1r-Cre/GCaMP3 mice after stimulation with the indicated treatments (featured traces here are from the same mouse). Tissue was stimulated with exendin-phe1 or exendin-asp3 at 10 nM for 10 minutes. Fluorescence intensity is reported as $\Delta F/F_0$, where ΔF is the background- and baseline-corrected fluorescence value, and F_0 is the average fluorescence signal in the 5 minutes prior to stimulation. Traces from individual regions of interest (ROIs), which represent individual cells, are in grey. The average fluorescence intensity for all cells is in colour. $n=4$ independent experiments, with 9-30 cells analysed per treatment. **(B)** As in (A) but with tissue re-challenged with 100 nM exendin-4 after acute stimulation with either exendin-phe1 or exendin-asp3 and 2 hours of recovery. $n=4$. **(C)** AUC of the average traces in the acute and re-challenge stimulations. Mann-Whitney U test with pairwise comparisons between exendin-phe1 and exendin-asp3. $*p<0.05$, $**p<0.01$. Data are presented as mean $\pm$ SEM. Non-parametric tests used given that individual neuronal responses to exendin-asp3 were not normally distributed. **(D)** Fluorescence intensity AUC values for individual cells pooled from the 4 experiments in the acute stimulation setting. Box plots indicate median value with whiskers denoting the 10th and 90th percentiles. Mann-Whitney U test, $\#p<0.0001$. **(E)** As in (D) but for the re-challenge stimulation setting.

5.3.6 Anorectic responses to biased GLP-1RAs after central administration

To probe the possibility that the differences in exendin-asp3's effects *in vitro/ex vivo* compared to its anorectic effects after peripheral administration are partly attributable to neurons *in vivo* being exposed to lower agonist concentrations, compounds were administered directly into the CSF via a lateral ventricle cannula, and their anorectic effects assessed. The selected dose (135 pmol/kg) was based, in part, upon a previous study where lateral ventricle delivery of 2.4 pmol/mouse of exendin-4 (equivalent to 96 pmol/kg assuming an average body weight of 25 g) resulted in a cumulative food intake reduction of 80-90% relative to vehicle at 6 hours [466]. Similar doses were also used to achieve physiological effects upon femoral arterial blood flow, heart rate and glucose utilisation [522], [523]. Moreover, given that the total CSF volume of a mouse is estimated at 35 μ l [524], a 135 pmol/kg dose equates to a CSF concentration of 115 nM (assuming uniform distribution and no backward flow of agonist from the CSF into the systemic circulation), which is similar to the 100 nM dose typically used *in vitro*. This is 12 000-fold higher than measured CSF levels after peripheral administration of exendin-4 (9.55 pM; though admittedly local concentrations at CNS neurons may considerably higher than when distributed throughout the CSF). ICV administration can thus be considered a test of supramaximal CNS GLP-1R stimulation, to determine whether agonist effects in *in vitro* pharmacological testing performed above can be recapitulated *in vivo*, given adequate dosing.

Indeed, after delivery of a high dose into the CSF, the anticipated bias-related differences between exendin-phe1 and exendin-asp3 were reinstated, with exendin-asp3 exhibiting a progressively lesser anorectic effect over time relative to exendin-4 and exendin-phe1 (Figure 5.6 A). This difference was maximal at 24 hours (Figure 5.6 B). Exendin-4 and exendin-phe1 exerted matched anorectic effects, contrasting with glycaemic effects, for which exendin-phe1 is typically superior. In line with the reasoning above, this might be explained by a combination of limited receptor density in neurons combined with exendin-phe1's reduced signalling efficacy, preventing the enhanced effects it exhibits in pancreatic β -cells from being recapitulated.

Importantly, control studies indicated that the observed anorectic effects of the agonists were mediated by centrally-located GLP-1Rs. Firstly, peripheral administration of exendin(9-39) at doses shown previously to antagonise GLP-1R-mediated anorexia [525], [526] failed to antagonise the anorectic effect of centrally-administered exendin-4 (Figure 5.6 D – in comparison to the appropriate control condition with vehicle injected centrally and exendin(9-39) injected peripherally). Secondly, exendin-4 was not detected in the peripheral circulation after central administration at 135 pmol/kg (Figure 5.6 F). By contrast, it was readily detectable after intraperitoneal injection at a dose that achieved comparable food intake reduction at the 1-hour mark (~50% reduction; *cf.* Figure 3.3 A). These data suggest that the anorectic effects of ICV delivered GLP-1RAs are centrally mediated, as previously stated by other authors [163], [180].

Interestingly, between 8 and 24 hours, exendin-4 and exendin-phe1 were still exerting anorectic effects (Figure 5.6 C). This is in contrast to after peripheral administration, where animals typically exhibit compensatory hyperphagia between 8 and 24 hours due to progressive renal clearance resulting in sub-anorectic plasma peptide concentrations, and meaning that counterregulatory orexigenic processes now dominate. This difference likely reflects the slower clearance of compounds from the CSF relative to the blood; additional steps of peptide movement from the brain parenchyma or CSF to the systemic circulation are required before renal clearance [395], [527], [528]. Sustained

concentrations in the CSF due to slower clearance likely contribute to exendin-asp3's progressively worse anorectic effects after ICV administration.

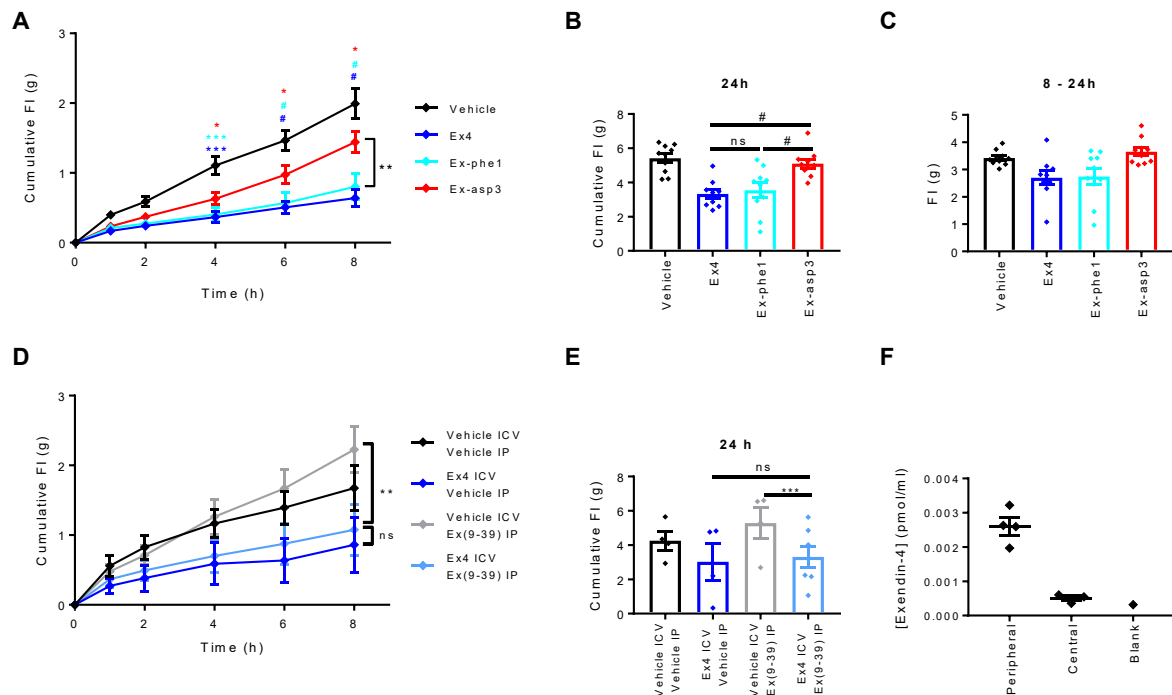


Figure 5.6. Anorectic responses after central administration of biased GLP-1RAs.

(A) Cumulative food intake in lean, male C57Bl/6 mice after administration of agonist (135 pmol/kg) via a lateral ventricle cannula at the indicated time points, $n=10/\text{group}$. Two-way repeat measures ANOVA with Tukey's test, with significance vs. saline shown, except for bracketed difference where significance between ex-phe1 and ex-asp3 at the 8h mark is indicated. (B) Cumulative food intake for mice in (A) at 24h. Two-way repeat measures ANOVA with Tukey's test. (C) Food intake for mice in (A) at between 8 and 24h. (D) Cumulative food intake in lean, male C57Bl/6 mice after administration of exendin-4 (135 pmol/kg) or vehicle via a lateral ventricle, and concomitant intraperitoneal administration of exendin(9-39) (200 nmol/kg) or vehicle, $n=4-6/\text{group}$. Two-way repeat measures ANOVA with Tukey's test. (E) Cumulative food intake for mice in (D) at 24h. Two-way repeat measures ANOVA with Tukey's test. (F) Plasma exendin-4 levels 1 hour after either peripheral (2 nmol/kg) or central (135 pmol/kg) administration of exendin-4, $n=3-4$. ** $p<0.01$, # $p<0.0001$. Data represented as mean $\pm$ SEM.

5.4 Discussion

The work described in this chapter investigated whether tissue-related differences between neurons and pancreatic β -cells influence the relative magnitude of response to the biased GLP-1RAs. The results of the previous chapter suggested that each compound had similar access to anorectic brain nuclei, thus prompting an alternative explanation for the divergence between the glucoregulatory and anorectic effects of the biased agonists after peripheral administration. Responses to the biased agonists were therefore assessed using *in vitro* and *ex vivo* models of arcuate nucleus neurons, and compared to concurrently gathered or previously reported [323] responses in the pancreatic β -cell line MIN6B1. A summary explanation, broadly supported by the results presented here but with caveats, for why the differences between the anorectic effects of biased GLP-1RAs are much smaller than for glucose regulation is that:

- A) Exendin-phe1 loses its usual “advantage” over exendin-4 as receptor density in neurons is not high enough for it to act as a full agonist and benefit from reduced desensitisation; and
- B) Typical systemic pharmacological doses *in vivo* are not high enough for desensitisation phenomena to adversely affect the anorectic effect of exendin-asp3 in neurons.

The distinctive characteristics of each compound are therefore negated, resulting in each performing similarly, with at most subtle differences in the kinetics of food intake recorded. With this in mind, it is helpful to consider the results presented in this chapter in the context of two questions:

1. What do they tell us about how neuronal *versus* β -cell GLP-1Rs respond to high doses of biased GLP-1RAs; and
2. How does this link to the physiological effects observed *in vivo* mediated by both neurons and pancreatic β -cells exposed to more typical agonist doses?

5.4.1 Neuronal and β -cell responses to high doses of biased GLP-1RAs

In vitro assays are typically either performed over a wide dose range for simple assays (e.g. some high throughput signalling readouts) or at single, usually maximal doses, particularly if the experiment is time- or resource-intensive. Higher concentrations, for which the efficacy-modulating effects of bias appear most obvious, are relevant to evaluation of biased GLP-1RAs. Previous work with exendin-4, exendin-phe1 and exendin-asp3 in MIN6B1 cells demonstrated that at high concentrations and over prolonged incubations, exendin-phe1 was the most insulinotropic and exendin-asp3 the least, matching the trafficking and bias profiles of each compound [323]. Here, as a secretory response could not be usefully obtained using mHypoA POMC/GFP3 cells, transcriptomic analysis was performed, with the rank order of agonist efficacy assessed as the number of differentially regulated genes when cells were stimulated with 100 nM agonist (a high dose). A similar study herein with MIN6B1 cells demonstrated the validity of this approach, as exendin-phe1 was found to be the most transcriptionally active, matching its insulin release profile. However, for mHypoA POMC/GFP3 cells, the results were reversed, with far more genes being up- or down-regulated with exendin-4 than with exendin-phe1.

It is proposed here that this reflects the failure of exendin-phe1 to engage a full acute signalling response in these cells, as demonstrated by the weak partial agonist activity demonstrated by the

acute cAMP assay (Figure 5.1, Table 5.1). As discussed in the results section above, recent work in the laboratory has shown that exendin-phe1 is a partial agonist for $G\alpha_s$ recruitment, and, given the considerably higher GLP-1R expression in MIN6B1 cells than mHypoA POMC/GFP3 cells (Figure 5.2 E), it follows that exendin-phe1 acting on β -cells is able to continually re-initiate large amplitude responses without constraints imposed by β -arrestin recruitment, leading to cumulative efficacy increases when compared to exendin-4. In contrast, in neurons, the low efficacy responses of exendin-phe1 even at high concentrations negate its presumed advantage of reduced desensitisation. In mHypoA POMC/GFP3 cells, the very low levels of GLP-1R expression magnified this effect to the point that the exendin-phe1 transcriptional response was lower than that of exendin-4 (Figure 5.2). However, the *in vivo* equivalent of this approach, wherein agonists were applied directly into the CSF at a dose predicted to achieve similar local concentrations to those used in the *in vitro* studies, showed that exendin-phe1 was as anorectic as exendin-4 (but not more so; Figure 5.6). This discrepancy may reflect relative levels of GLP-1R expression in primary tissue *versus* immortalised cell lines (potentially artificially lowered in the latter), or indeed more complex aspects of *in vivo* neurophysiology.

In contrast, exendin-asp3's anticipated time-dependent signalling deficit relative to exendin-4 or exendin-phe1, typified by previously reported insulin secretory responses in pancreatic β -cells, was generally recapitulated when neurons were exposed to high agonist concentrations through the combined approaches used in this chapter. Despite its high signalling efficacy acutely, exendin-asp3 did indeed induce greater GLP-1R desensitisation in both mHypoA POMC/GFP3 cells and in ARC brain slices. Crucially, this effect could be recapitulated *in vivo* after exposing CNS neurons to supramaximal concentrations by ICV administration, with exendin-asp3 exhibiting progressively weaker anorectic effects relative to exendin-phe1. Moreover, the transcriptomic responses to prolonged stimulation showed exendin-asp3 to be the least effective ligand, especially compared to exendin-4.

5.4.2 Link to physiological effects of peripherally administered biased GLP-1RAs

The findings discussed in Section 5.4.1 pertain mainly to responses to agonist doses *circa* 100 nM sustained over prolonged periods and are relevant to an understanding of how the GLP-1R responds when maximally stimulated. However, it should be recognised that typical doses of exendin-4 and biased GLP-1RAs may not achieve concentrations as high as this, either in the systemic circulation or in the CNS. Indeed, a plasma concentration in the high picomolar range was recorded in rats in this project 1 hour after administration of a high dose of 100 nmol/kg exendin-4 (Figure 4.17). On the other hand, peak plasma exendin-4 concentrations >10 nM have been recorded in mice [444], [445], albeit with doses somewhat higher than typically used for physiological studies. It should be borne in mind that exendin-4 assays are not standardised and absolute reported concentrations may be subject to significant variability, precluding uncritical comparisons between different studies. Agonist and temporal comparisons within the same study should be reliable however.

If it is accepted that most of the biased GLP-1RA doses used for physiological testing in Chapter 3 are unlikely to achieve concentrations of agonist approaching those used for supramaximal *in vitro* testing, even accounting for assay variability, it is likely to be of significant importance that the concentration range over which neurons respond is considerably higher than for β -cells, as demonstrated both for acute cAMP measurements in mHypoA POMC/GFP3 and MIN6B1 cells (Table

5.1), and for acute anorectic *versus* glycaemic effects in mice (Table 3.1). As depicted in Figure 5.7, moderate agonist doses *in vivo* may already be sufficient to maximally stimulate β -cells (for example, see Figure 3.4 D, where ~ 1 nmol/kg of each agonist provides a maximal glucose-lowering response during acute IPGTT at 20 minutes). Therefore, responses measured in the *in vitro/ex vivo* studies in the present chapter at 100 nM are highly likely to also engender a maximal response at β -cells (Figure 5.7). Admittedly, the situation is not identical as the *in vitro* system is closed, whereas agonist concentration changes dynamically *in vivo*.

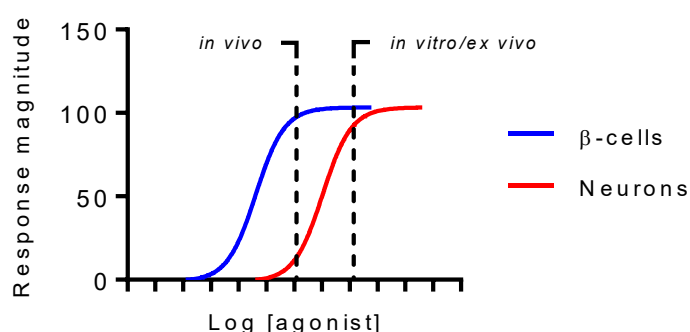


Figure 5.7. Schematic explaining the differential responsivity of pancreatic β -cells and neurons.

The doses to which pancreatic β -cells respond are notably lower than that for neurons, as indicated by the leftward shift of the theoretical dose-response curve for β -cells *versus* neurons. After peripheral agonist administration *in vivo*, only low plasma and CNS agonist concentrations are achieved (dotted line denoted “*in vivo*”), thus resulting in maximal signalling and desensitisation responses in β -cells but not in neurons. By contrast, at the considerably higher concentrations applied in *in vitro/ex vivo* assays (dotted line denoted “*in vitro/ex vivo*”) are able to induce maximal responses at both cell types (provided that the ligand is a full agonist).

Contrasting with this, at doses within the gluco-discriminatory range (0.1 – 10 nmol/kg), the reduced sensitivity of GLP-1R-expressing neurons means they are not likely to be maximally activated (Figure 5.7), which means that the high-dose results described in the present chapter are less easy to directly extrapolate to the physiological responses observed. Some subtle evidence of increased waning of anorectic response with exendin-asp3 has been observed *in vivo*, mainly at higher doses (e.g. Figure 3.3 A and Figure 3.4 F), but the magnitude of this difference is much smaller than the progressive nullification of the exendin-asp3 anorectic response when delivered ICV (Figure 5.6) or at the transcriptional level in mHypoA POMC/GFP3 cells (Figure 5.2). Crucially though, given exendin-phe1’s inability to elicit a maximal signalling response in neurons even at the high agonist concentrations used *in vitro/ex vivo* (the reasons for which are discussed above in Section 5.4.1), it therefore follows that it is also unable to do so at the lower plasma and CNS concentrations achieved after peripheral administration *in vivo*. Its reduced signalling efficacy in neurons *in vivo* is likely a major contributor to the fact that it fails to show enhanced anorectic efficacy relative to the other agonists.

5.4.3 Experimental Caveats

It should be acknowledged that the models used here are gross simplifications of *in vivo* physiology. mHypoA POMC/GFP3 cells are a model of ARC POMC/CART neurons that, whilst important in mediating the anorectic effects of GLP-1RAs [179], [465], [466], are likely not the only neuronal

population mediating the anorectic effects of the biased GLP-1RAs tested here. Monoculture of mHypoA POMC/GFP3 cells therefore omits input from phenotypically distinct intra- and inter-ARC neuronal populations. Moreover, *in vivo* three-dimensional network connectivity is not recapitulated by monolayers of these cells. *Ex vivo* brain slices represent an improvement upon immortalised cell lines, given the better preservation of ARC neuronal population heterogeneity and a degree of intra-ARC connectivity. However, inter-regional neurocircuitry that may be important for the manifestation of physiological responses is lost in slice culture, including DVC-hypothalamus connectivity.

An additional caveat of *in vitro* and *ex vivo* assays, discussed extensively above, is that agonist concentration is often considerably higher than that *in vivo*. In fact, the results obtained here directly caution against the over-interpretation of results in such assays for this very reason. The differences in exendin-asp3's anorectic effects after peripheral *versus* central administration suggest that its signalling properties are affected by the local concentrations of agonist to which anorexigenic neurons are exposed.

5.4.4 Future work

Despite these caveats, *in vitro* and *ex vivo* assays provide tractable systems for the study of neurons and β -cells and, indeed, both previous studies [323] and the results here report parallels between the *in vitro* and *in vivo* effects of biased GLP-1RAs at both β -cells and neurons. However, a number of additional experiments could be conducted to reinforce the existence of tissue bias, and interrogate its underlying causes.

Firstly, in view of the influence of ligand concentration, some of the experiments performed in the current chapter would be repeated at lower agonist doses to model *in vivo* systemic exposures. Here it might be anticipated that, in β -cells, agonist-specific response patterns would be preserved as concentrations remain towards the top of the response curve, but in neurons or after ICV administration *in vivo*, differences in responses between agonists would converge as the impact of desensitisation is reduced. It would be impractical or unfeasible to perform full dose ranges for resource-intensive studies such as RNAseq or ICV cannulations, but qPCR could be used to establish dose ranges for transcriptional markers identified herein. In β -cells, both cAMP accumulation and insulin secretion could be detected after short- and long-term incubations, allowing detailed characterisation of agonist effects over time [323]. However, prolonged cAMP responses could not be detected in mHypoA POMC/GFP3 cells, and, as just noted, RNAseq would be impractical to use for time course analysis. Neurons rely upon the release of neuropeptides or neurotransmitters for signalling, and thus levels of neuropeptides released by POMC/CART neurons *in vivo* after agonist stimulation could be assessed at different time points. This would provide a parallel to the study of insulin secretion in β -cells, and allow study of how neuronal responses to the agonists diverge over time. Unfortunately, α -MSH release could not be detected with mHypoA POMC/GFP3 cells, but it is possible that alternative neuropeptides could be measured.

The mHypoA POMC/GFP3 cells are derived from POMC/CART ARC neurons; however, only 10% of this population express the GLP-1R [83]. To overcome this, primary neurons could be isolated from *Glp1r-Cre/GCaMP3* mice and Ca^{2+} responses studied in GLP-1R-expressing neurons (differentiated from GLP-1R-negative neurons upon the basis of their fluorescence). The use of primary cells would better recapitulate neuronal biology relative to an immortalised cell line, and would allow both

hypothalamic and DVC GLP-1R-expressing neurons to be isolated and studied separately. Moreover, pancreatic islets could be isolated from matched mice and Ca^{2+} responses to the agonists studied in parallel.

The described studies would help confirm the existence of tissue bias by comparing neuronal and β -cell responses. To investigate its mechanistic basis, relative levels of GLP-1R, β -arrestin and G-protein expression could be assessed in primary neuronal and islet tissue. To obtain pure GLP-1R-expressing cultures, neurons and β -cells from *Glp1r-Cre/GCaMP3* mice could be sorted by fluorescence activated cell sorting (FACS), and receptor and effector expression subsequently assessed by qPCR, RNAseq, or to take account of likely heterogeneity [83], single cell RNAseq. Additionally, if exendin-phe1's reduced signalling efficacy is linked to low GLP-1R expression in neurons, transfecting mHypoA POMC/GFP3 cells or primary neurons with high levels of GLP-1R should hypothetically re-establish it as a full agonist in these cells. Admittedly, DNA transfection efficacy in neurons (particularly primary cultures) is noted to be low [529], so alternative approaches such as viral vectors, nucleofection or mRNA transfection may be required.

A more comprehensive assessment of the relationship between GLP-1R expression and the signalling response of the agonists could be conducted by transfecting increasing levels of *Glp-1r* DNA into cells that do not endogenously express the receptor (e.g. HEK or CHO cells) and measuring downstream responses after prolonged incubation (e.g. cAMP accumulation or a cAMP response element [CRE]-linked luciferase reporter). The GLP-1R expression level-intracellular response relationship for exendin-phe1 would be expected to be distinct from exendin-4 and exendin-asp3, i.e. at low levels of GLP-1R expression exendin-phe1 would be predicted to act as a low efficacy partial agonist, but beyond a certain threshold, sufficient receptor may be present to support increased efficacy via reduced desensitisation with this agonist. Given that the stoichiometric ratio of cellular signalling components may differ between cell types [342], [392] and has been shown to affect cellular responses [530], conducting these experiments in pancreatic β -cell [531], [532] and neuronal lines with endogenous GLP-1R expression knocked out/down would be a further improvement. Of course, transfections in primary cells with the GLP-1R depleted would represent the optimal experimental design but would likely be impractical.

5.4.5 Summary and relevance

In summary, the results in this chapter evidence differences in response magnitude to the biased GLP-1RAs in pancreatic β -cells *versus* neurons. In particular, exendin-phe1's differential signalling efficacy in the two cell types – acting as a full agonist in pancreatic β -cells but a partial agonist in neurons – appears to explain why its superior, time-dependent anti-hyperglycaemic effects fail to translate into commensurate increases in anorectic efficacy relative to the other two agonists. In turn, differences in exendin-phe1's efficacy appear related to differential levels of GLP-1R expression and signal amplification in the $\text{G}\alpha_s$ pathway in β -cells *versus* neurons. Admittedly, these may not be the only factors underlying the divergence between the anorectic and glucoregulatory effects of the biased GLP-1RAs, and further investigation is warranted.

More broadly, the work of this chapter provides evidence that cellular context can affect the response to biased agonists. This general phenomenon is referred to as “system bias” when applied to the screening for biased agonists in different immortalised cell lines. Tissue bias can therefore be viewed

as the physiologically relevant equivalent given that multiple GLP-1R-expressing tissues – each with phenotypically distinct cells – are exposed to systemically administered GLP-1RAs. Understanding the impact of tissue-specific GLP-1R signalling is important given that both the beneficial effects (insulinotropism and weight loss) and side effects (e.g. nausea, vomiting) of GLP-1RAs are mediated by β -cells and neurons. Optimising responses at both tissues is therefore critical for the therapeutic application of biased GLP-1RAs.

6 Physiological Effects of Acylated Biased GLP-1RAs

6.1 Introduction

6.1.1 Acylation as a means for half-life extension

This chapter reports the cellular and physiological testing of pharmacokinetically improved iterations of exendin-4, exendin-phe1 and exendin-asp3 featuring the covalent addition of an acyl chain (“acylation”) to extend *in vivo* half-life. Acylation is a well-established peptide modification that prolongs biological action by promoting peptide self-aggregation and binding to serum albumin [235]. Self-aggregation results in the formation of a depot at the site of subcutaneous injection and slows absorption into the systemic circulation. Once in the systemic circulation, binding to albumin reduces renal clearance due to the large size of the peptide-albumin complex, and also sterically hinders against enzymatic degradation [235]. Crystallographic studies have revealed that the fatty acids C10, C12, C14, C16 and C18 bind to human serum albumin at seven sites distributed across the protein [533]. Albumin represents an attractive binding partner given its high plasma concentration, broad biodistribution and tissue penetration, and long half-life of several weeks. In addition to its size being above the renal filtration threshold, its long half-life is also attributable to its ability to bind the neonatal Fc receptor (FcRn) in a pH-dependent manner [534]. High-affinity binding of albumin to the FcRn promotes recycling of the complex back to the plasma, thereby protecting against lysosomal degradation [535]. In the plasma it dissociates from the FcRn due to its low affinity for the receptor at neutral pH. Although this mechanism of avoiding intracellular degradation may be relevant to the half-life extension of albumin-bound therapeutics in general, direct evidence for this is yet to be demonstrated for GLP-1RAs [235].

Acylation as an approach for extending the bioactivity of peptide therapeutics has been extensively investigated in the T2DM field over the past two decades. In 1998, Eli Lilly generated a form of GLP-1 conjugated to a C8 fatty acid and with His7, Arg26 amino acid modifications [536]. Despite an extended plasma half-life, its therapeutic application was limited by its physical instability and propensity for self-association [259]. The first clinically approved acylated peptide therapeutic was insulin detemir (Levemir, Novo Nordisk) – a C14-conjugated form of insulin with a half-life of 5-7 hours (*versus* 4-6 minutes for native insulin) that facilitated once daily-dosing [235]. The latest, improved iteration is insulin degludec (Tresiba, Novo Nordisk), a C16-conjugated insulin analogue with a half-life of around 25 hours [537]. In addition to these long-acting insulin analogues, Novo Nordisk have produced acylated GLP-1RAs liraglutide (Victoza) and semaglutide (Ozempic), the development of which are discussed below.

6.1.2 The development of liraglutide and semaglutide: considerations for the acylation of GLP-1RAs

The development of liraglutide and semaglutide has provided considerable insight into the factors governing the pharmacodynamics and pharmacokinetics of acylated GLP-1RAs. These include the position of peptide derivatisation, the length of the fatty acid chain, the nature of the fatty acid (acid or diacid, inclusion of polar elements, bulkiness of the chain), the number of fatty acid chains, and the nature of the linker/spacer.

Liraglutide, based upon native human GLP-1, has a C16 fatty acid conjugated to Lys26 via a γ -glutamic acid spacer and an Lys to Arg substitution at position 34 [235]. During development,

investigation into the effect of the position of lysine-mediated derivatisation revealed C-terminus acylation to have minimal effects upon receptor potency [538]. Exceptions to this were at positions 28 and 29. By contrast, N-terminus acylation at positions 7, 10, 12, 13, and 15 all resulted in a severe loss of potency [538], consistent with the results of an earlier systematic alanine (Ala) scan of the GLP-1 sequence [539] and more recent structural studies [377] evidencing the criticality of the N-terminus to receptor binding and activation. Despite a number of benign C-terminus conjugation points, the endogenous lysine at position 26 was chosen to minimise amino acid substitutions and therefore both simplify the synthesis process as well as avoid immunogenicity responses (the likelihood of which are increased with reduced homology to human GLP-1) [259].

Considering the length of the fatty acid chain, *in vivo* half-life was found to be positively correlated with chain length due to increased albumin binding; however, receptor potency was adversely affected by fatty acid chains longer than 16 carbon atoms [540]. High affinity binding to albumin with long fatty acid chains reduces the free fraction of agonist capable of activating the GLP-1R, accounting for the decrease in potency [259]. The GLP-1RA Albiglutide takes this to the logical extreme, with two copies of a GLP-1 analogue covalently conjugated to human albumin [235]. Although significant half-life protraction is obtained, very high doses of Albiglutide are required to achieve relevant clinical efficacy [259]. Conjugation of fatty diacids (i.e. with two carboxylic acid functional groups) to liraglutide-like compounds was slightly less well tolerated than conjugation to monoacids, with diacids longer than 14 carbon atoms resulting in loss of activity at the GLP-1R [538]. Use of diacids, however, increased binding to albumin and solubility [538]. Conjugation of C14 or C16 fatty acids at both endogenous lysines in the GLP-1 sequence (positions 26 and 34 i.e. multiple fatty acid chains) resulted in marked loss of potency [538]. Other modifications to the acyl chain – the addition of polar elements or increasing its bulkiness – led to variable changes upon half-life protraction and receptor potency, and were disadvantageous overall [540].

A spacer, or linker, is used to conjugate the fatty acid to the peptide sequence in order to reduce steric hindrance between the two entities and retain biological activity. Accordingly, removal of the spacer for liraglutide-like compounds resulted in significantly reduced potency without affecting half-life protraction [540]. Several amino acid-derived spacers – β -alanine, γ -aminobutyric acid, and γ -glutamic acid (γ -Glu) – all yielded similarly potent compounds for the same length fatty acid, though the γ -Glu spacer resulted in the longest *in vivo* half-life [538], [540]. By contrast, the use of alternative, non-amino acid-derived spacer triethylenglycol notably reduced potency whilst not affecting half-life [540].

Hence, with these considerations in mind, the design of liraglutide – with a 16C fatty acid attached via γ -Glu at Lys26 – achieved high receptor potency with pharmacokinetics optimal for once-daily dosing [259]. Semaglutide was subsequently developed as a once-weekly formulation, with further modifications to both peptide sequence and fatty acid to extend half-life beyond that achieved for liraglutide [259]. An Ala to α -aminoisobutyric (Aib) substitution at position 8 was introduced to increase resistance to DPP-IV-mediated degradation [259]. A longer, C18 fatty diacid conjugated at Lys26 via a spacer composed of γ -Glu and two 8-amino-3,6-dioxaoctanoic acid moieties (γ -Glu-2xOEG) was found to be optimal to obtain high albumin binding and GLP-1R potency [259]. The cumulative effect of these modifications is a half-life of 56 hours (extended from liraglutide's 10 hours) [235]. Interestingly, GLP-1R potency is also improved for semaglutide vs. liraglutide despite the longer acyl chain and use of a diacid [236]. This effect is attributable to the optimised γ -Glu-2xOEG spacer, as

loss of potency relative to liraglutide observed with C14 to C20 diacids conjugated via a γ -Glu spacer is recovered by introduction of a γ -Glu-2xOEG spacer [236]. Thus semaglutide represents a second generation, pharmacodynamically and pharmacokinetically improved form of liraglutide.

6.1.3 The design of acylated exendin-4-based biased GLP-1RAs

Upon this knowledge base, the exendin-4-based peptides tested in this chapter were modified by the addition of a C16 fatty diacid attached to the C-terminus (additional Lys at position 40). A C16 chain was chosen as a suitable compromise between achieving high potency at the GLP-1R and an extended pharmacokinetic profile, with the expectation that the protease-resistant exendin-4 amino acid sequence should also contribute to the latter. C-terminus conjugation was used given its benign effect for GLP-1 analogues, and a previous report that conjugation of hydrophobic fluorophores to the exendin-4 sequence at position 40 led to little effect upon potency [442]. Additionally, for certain fatty acids, acylation of human GLP-1 at more extreme C-terminus positions (i.e. position 34 or 38 vs. 26) actually increases receptor potency [538]. Conjugation of a diacid with no spacer was trialled given that the same modification to GLP-1 at positions 36 or 38 also increased potency relative to native GLP-1 [538]. This highlights the importance of derivatisation position, as omission of a spacer at position 26 for either monoacids or diacids results in a loss of potency [538], [541]. Additionally, use of a diacid rather than a monoacid should lead to greater half-life protraction. It should be borne in mind that, although previous reports on GLP-1 acylation provide useful guidance, the notable differences in peptide sequence between GLP-1 and exendin-4 likely mean that the actual effect of acylation upon the exendin-4-based compounds here cannot be precisely predicted.

6.1.4 Aims

1. To determine the effects of acylation of exendin-4-based biased GLP-1RAs upon cellular properties *in vitro*, specifically:
 - a. Biased signalling at the GLP-1R;
 - b. GLP-1R internalisation and recycling;
 - c. GLP-1R binding kinetics; and
 - d. Insulin secretion in pancreatic β -cell lines.
2. To determine the effects of acylation of exendin-4-based biased GLP-1RAs upon physiological responses *in vivo*, specifically:
 - a. Glycaemic and anorectic responses in chow-fed mice; and
 - b. Glycaemic and anorectic responses in diet-induced obese mice.

6.2 Results

6.2.1 Quantitation of biased signalling of acylated GLP-1RAs

Given that acylation can alter the pharmacological properties of GLP-1RAs [538], [540], cAMP accumulation and β -arrestin-2 recruitment was assessed for the acylated peptides, and bias subsequently quantified. Results are summarised in Figure 6.1 and Table 6.1. cAMP potency and efficacy for exendin-4-acyl and exendin-asp3-acyl were essentially unchanged from their unmodified counterparts. However, exendin-phe1-acyl had significantly decreased potency (4.89-fold higher EC_{50}) relative to exendin-phe1. The increased sensitivity of exendin-phe1 to modifications as compared to exendin-4 and exendin-asp3 has been observed previously with FITC conjugation at lysine 12 [323], as well as in Chapter 4 with sCy7 conjugation to pra12. Given the difference in both conjugation point and functional group used in this chapter, it appears that phenylalanine substitution at position 1 in the exendin-4 sequence increases overall sensitivity to any deviation from the unmodified agonist structure.

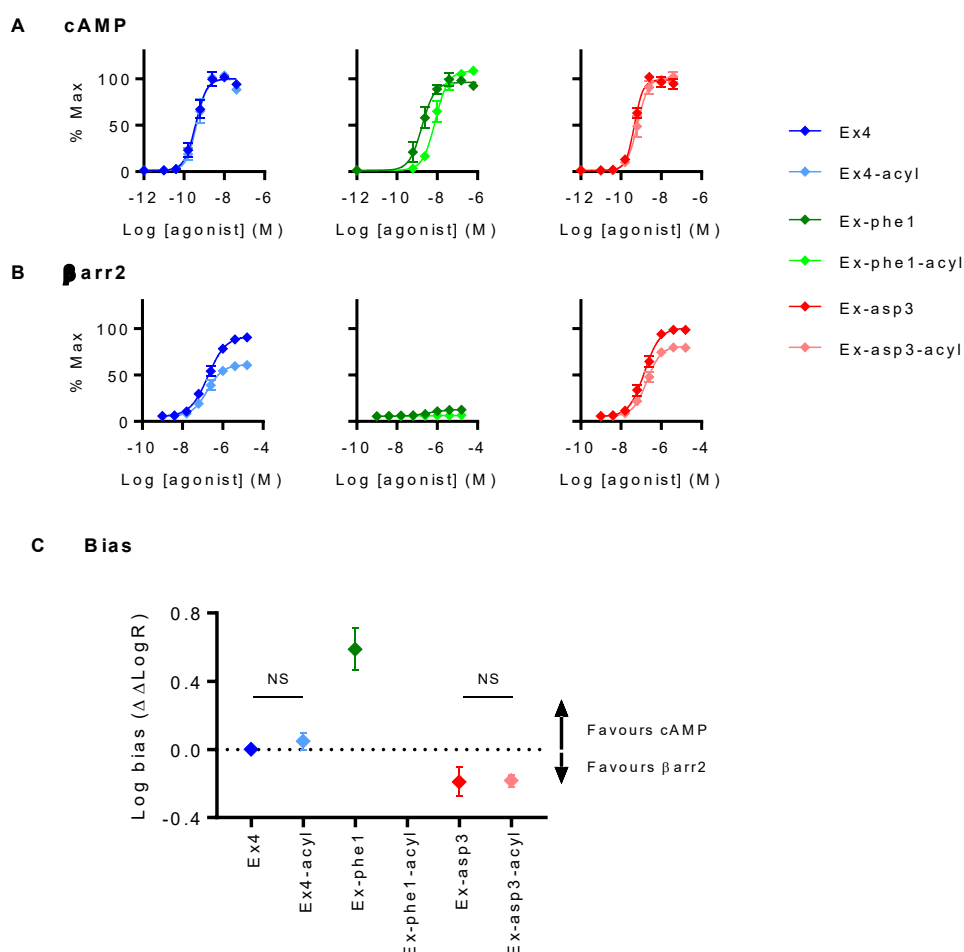


Figure 6.1. Quantitation of biased signalling of acylated GLP-1RAs.

(A) cAMP responses to unmodified and acylated forms of exendin-4, exendin-phe1 and exendin-asp3 in PathHunter CHO-GLP-1R β -arrestin-2 reporter cells, 30 min incubation. Data are expressed as a percentage of the maximal response in the assay with four-parameter logistic fit of data. $N=5$, data represented as mean $\pm$ SEM. **(B)** As in (A) but for β -arrestin-2 recruitment. **(C)** Quantitation of biased signalling for data in (A) and (B) with $\log(\tau/K_A)$ values for each agonist normalised to reference agonist exendin-4. One-way repeat measures ANOVA. Data represented as mean $\pm$ SEM.

Interestingly, all acylated agonists showed reduced maximal β -arrestin-2 recruitment relative to their unmodified forms. This translated to only minor changes in reported bias factor (larger for exendin-4-acyl than exendin-asp3-acyl), likely reflecting the fact that the model used for bias quantitation, in effect, places greater emphasis upon changes in EC_{50} than those in E_{max} (or at least it can appear this way with the logarithmic x-axis scale which minimises the visual impact of what can still be significant potency shifts). Note that, given the non-sigmoidal β -arrestin-2 responses for exendin-phe1-acyl, LogR and therefore a bias factor value could not be derived. Alternative approaches, such as using the ligand as an antagonist, could be trialled if it was considered essential to numerically derive a bias factor [542]. However, the broad similarity of its signalling responses to exendin-phe1 make it highly likely that it is also G-protein biased, with the observed decrease in cAMP potency likely counterbalanced by reductions in potency and efficacy for β -arrestin-2 recruitment. Thus, the pattern of bias between the three agonists is unchanged by acylation, though its overall effect appears to be that of reduced β -arrestin-2 recruitment.

Peptide	cAMP				
	pEC ₅₀ (nM)	$\Delta\log EC_{50}$	EC ₅₀ Ratio	E _{max} (%)	Ratio
Ex4	9.42 ± 0.13			100 ± 4.75	
Ex4-acyl	9.38 ± 0.13	0.03 ± 0.01	1.08	99.8 ± 1.83	1.00
Ex-phe1	8.81 ± 0.16			96.6 ± 1.70	
Ex-phe1-acyl	8.12 ± 0.10 #	0.69 ± 0.05	4.89	109 ± 3.18	1.12
Ex-asp3	9.32 ± 0.06			98.2 ± 3.17	
Ex-asp3-acyl	9.18 ± 0.13	0.14 ± 0.03	1.37	102 ± 3.44	1.04
Peptide	β -arrestin-2				
	pEC ₅₀ (nM)	$\Delta\log EC_{50}$	EC ₅₀ Ratio	E _{max} (%)	Ratio
Ex4	6.74 ± 0.12			97.9 ± 1.71	
Ex4-acyl	6.76 ± 0.11	0.02 ± 0.01	1.03	65.1 ± 3.18 #	0.67
Ex-phe1	6.21 ± 0.29			14.6 ± 1.61	
Ex-phe1-acyl	ND	ND	ND	4.09 ± 11.74	0.28
Ex-asp3	6.85 ± 0.11			107 ± 1.04	
Ex-asp3-acyl	6.72 ± 0.09	-0.13 ± 0.02	1.34	86.4 ± 3.26 #	0.81

Table 6.1. Potency and efficacy of acylated GLP-1RAs for cAMP accumulation and β -arrestin-2 recruitment.

Values are derived from Figure 6.1. " $\Delta\log EC_{50}$ " is the difference in $\log EC_{50}$ values between unmodified and acylated counterparts. "EC₅₀ Ratio" describes the ratio between the average EC₅₀ values for unmodified and acylated counterparts. "Ratio" denotes E_{max} value relative to that of the equivalent unmodified compound. E_{max} values normalised to global maximum in individual experiments. Significance reported in comparison to unmodified peptide e.g. ex4 vs. ex4-acyl. One-way randomised block ANOVA with Tukey's test. #p<0.0001. Data reported as mean ± SEM from 5 independent experiments.

6.2.2 Measurement of GLP-1R internalisation

The results above suggest that acylation leads to reduced β -arrestin-2 recruitment for the tested peptides. This may manifest as changes in GLP-1R internalisation given the demonstrated role for β -arrestins in this event [323]. Internalisation was therefore measured in real-time using diffusion-enhanced resonance energy transfer (DERET), an assay based upon Förster resonance energy transfer (FRET) between N-terminally labelled SNAP-GLP-1R and extracellular buffer containing fluorescein [323], [327]. In the assay, the SNAP-tag of surface GLP-1Rs is covalently labelled with BG-Lumi4-Tb, a small benzyl guanine (BG) substrate coupled to donor fluorophore Lumi4-Tb that has several emission peaks (major peak at 490 nm, smaller peaks at 548, 587 and 620 nm [543]). Importantly, the long-lived nature of Lumi4-Tb's fluorescence allows signal to be read after a delay, omitting short-lived autofluorescence from culture plates and thus improving signal-to-noise ratio. This principle is referred to as time resolved fluorescence (TRF).

When SNAP-bound Lumi4-Tb is excited at 350 nm, fluorescence emitted at 490 nm results in FRET to fluorescein that in turn emits signal at 520 nm, and quenches Lumi4-Tb's 620 nm emission peak. After agonist-induced receptor internalisation, FRET cannot occur between Lumi4-Tb and fluorescein, leading to reduced 520 nm signal and increased 620 nm signal. The ratio between the two wavelengths (620/520 nm) provides a quantitative measure of receptor internalisation, such that its magnitude correlates positively with the level of internalisation. Serial measurements allow for real-time monitoring of internalisation; this can be conducted at various doses and a dose response curve constructed from the area under the curve of the individual traces to derive values for agonist potency and efficacy. The assay principle is summarised in Figure 6.2.

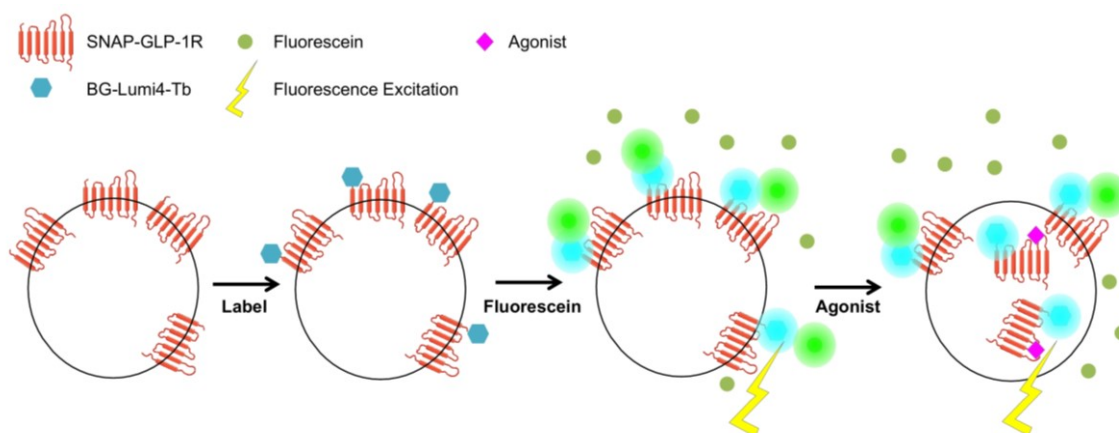


Figure 6.2. DERET Internalisation Assay Principle

As previously observed [323], exendin-4 and exendin-asp3 induced rapid and extensive GLP-1R internalisation, whereas exendin-phe1 caused a far slower loss of cell surface GLP-1Rs (Figure 6.3). This overall pattern was matched for the acylated agonists, though with maximal internalisation induced by exendin-4-acyl was significantly lower than that by exendin-4 (Table 6.2). This marries well with the reduced β -arrestin-2 recruitment seen for exendin-4-acyl (Figure 6.1) when considering the canonical role of β -arrestins in receptor internalisation. Intriguingly, acylation also caused a 2-fold (though non-significant) increase in potency for exendin-4 and exendin-asp3 in DERET internalisation assays, despite the reduced efficacy for exendin-4-acyl in this assay, and for β -arrestin-2 recruitment for both peptides. The significance of these observations is not clear. GLP-1R internalisation was also

studied by confocal microscopy, though the subtle differences in internalisation reported by DERET assays were not observable (Figure 6.4), likely reflecting the greater sensitivity of the latter assay.

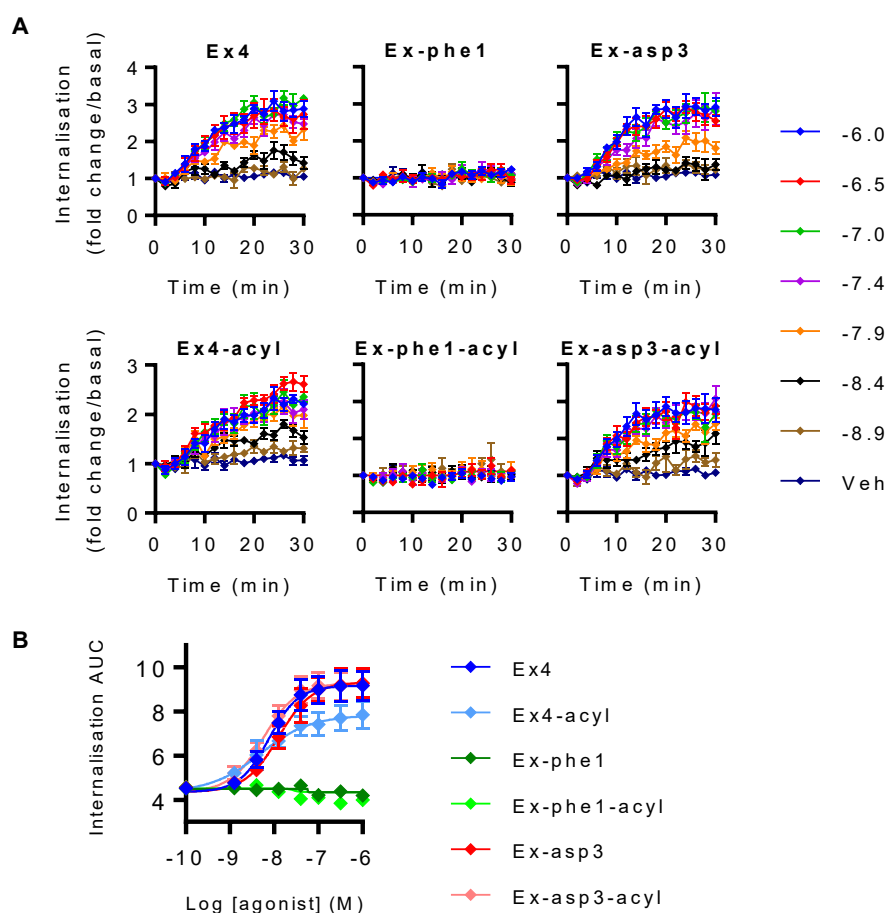


Figure 6.3. Real time GLP-1R internalisation after stimulation with acylated GLP-1RAs in HEK293-SNAP-GLP-1R cells.

(A) Internalisation time course in HEK293-SNAP-GLP-1R cells, with unmodified and acylated GLP-1RAs administered at the various indicated concentrations. FRET ratio read every 2 minutes for 30 minutes. Results are expressed after normalisation to basal signal read for 10 minutes prior to agonist addition, $n=5$. **(B)** Dose response for GLP-1R internalisation as derived from total AUC measurements from data in (A). Four-parameter logistic fit applied to data. Data represented as mean $\pm$ SEM.

Peptide	DERET				
	pEC ₅₀ (nM)	$\Delta\log\text{EC}_{50}$	EC ₅₀ Ratio	E _{max} (%)	Ratio
Ex4	8.08 $\pm$ 0.06			99.2 $\pm$ 0.91	
Ex4-acyl	8.38 $\pm$ 0.23	-0.30 $\pm$ 0.09	0.51	84.3 $\pm$ 1.75 #	0.85
Ex-phe1	ND			50.7 $\pm$ 3.57	
Ex-phe1-acyl	ND	ND	ND	52.4 $\pm$ 3.77	1.03
Ex-asp3	7.86 $\pm$ 0.07			101 $\pm$ 1.08	
Ex-asp3-acyl	8.22 $\pm$ 0.08	-0.36 $\pm$ 0.03	0.43	101 $\pm$ 0.34	1.00

Table 6.2. Potency and efficacy values for acylated GLP-1RAs for GLP-1R internalisation in HEK293-SNAP-GLP-1R cells.

Values are derived from data in Figure 6.3. “ $\Delta\log EC_{50}$ ” is the difference in $\log EC_{50}$ values between unmodified and acylated counterparts. “ EC_{50} Ratio” describes the ratio between the EC_{50} values for unmodified and acylated counterparts. “Ratio” denotes E_{max} value relative to that of the equivalent unmodified compound. E_{max} values normalised to global maximum in individual experiments. Significance reported in comparison to unmodified peptide e.g. ex4 vs. ex4-acyl. One-way randomised block ANOVA with Tukey’s test. # $p < 0.0001$. Data reported as mean $\pm$ SEM from 5 independent experiments.

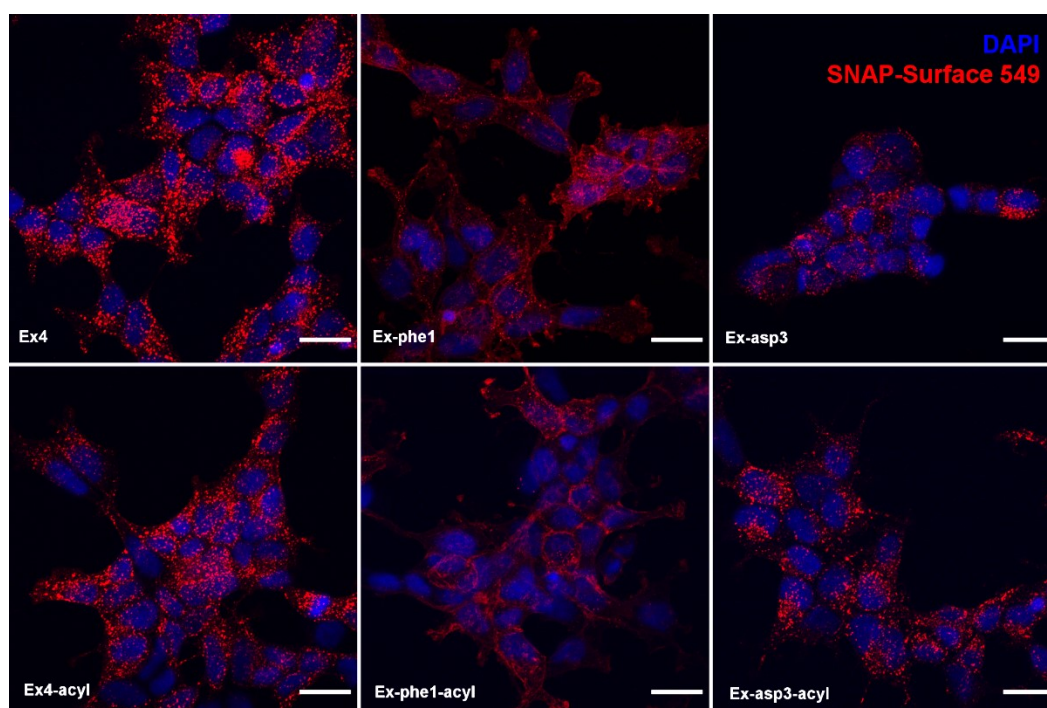


Figure 6.4. GLP-1R internalisation in HEK293-SNAP-GLP-1R cells.

Representative maximum intensity projection confocal images indicating internalisation of SNAP-GLP-1R labelled with SNAP-Surface 549 (red) after 30 minutes of agonist stimulation at 100 nM in HEK293-SNAP-GLP-1R cells. n=1 experiment, scale bar denotes 20 μm .

6.2.3 Measurement of GLP-1R recycling

The assessments of internalisation used above report only net cell surface loss of GLP-1R as they are unable to discriminate between the processes of receptor internalisation and recycling. A proportion of detected membrane-located receptor will have been internalised and subsequently recycled, but will be indistinguishable from the receptor fraction that did not internalise. To overcome this confounder and directly assess receptor recycling dynamics, a time-resolved FRET (TR-FRET)-based assay with reversible labelling of SNAP-tagged GLP-1Rs was conducted. In the assay, SNAP-tag was labelled with a cleavable form of BG-Lumi4-Tb that contains a reducible disulphide bond between the benzyl guanine and Lumi4 moieties (BG-SS-Lumi4-Tb). After agonist stimulation, residual surface receptors were stripped of label using membrane-impermeable reducing agent sodium 2-sulfanylethanesulfonate (MesNa) so that signal could derive only from internalised receptors. Lumi4-Tb fluorescence was read at 616 nm at this point to measure receptor

internalisation. Importantly, these steps were conducted with cells in ice-cold reagents to cause endocytic arrest. Cells were then returned to physiological temperature (37°C) to allow for receptor recycling and were incubated with LUXendin645 [381], a Cy5-tagged form of exendin(9-39) that acts as the assay's fluorescence acceptor. As Lumi4-Tb-labelled GLP-1R is recycled to the plasma membrane and bound by LUXendin645, FRET occurs between the two fluorophores given Lumi4-Tb's emission peak at 621 nm, resulting in 647 nm signal emission from LUXendin645's Cy5 moiety. The 647 nm to 616 nm signal ratio thus positively correlates with levels of recycled GLP-1R, and was measured serially to monitor recycling in real time. Of note, the use of GLP-1R antagonist LUXendin645 has an advantage relative to fluorescein: whilst the latter relies upon Brownian motion for fluorescein and receptor-associated Lumi4-Tb to come into proximity for FRET to occur, LUXendin645 binds directly to the receptor with high affinity, resulting in strong TR-FRET signal. The assay principle is summarised in Figure 6.5.

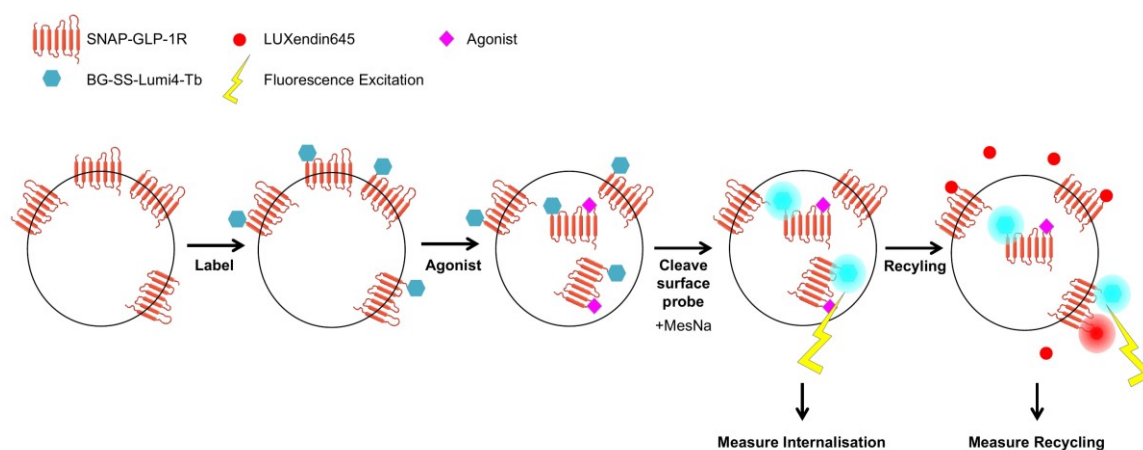


Figure 6.5. Recycling assay principle.

In keeping with the measurements of internalisation above (Figure 6.3), extensive internalisation of exendin-4, exendin-asp3 and matched acylated forms was observed (Figure 6.6 A). The small but significant difference in internalisation between exendin-4 and exendin-4-acyl detected in DERET assays was not recapitulated here, though this is likely due to internalisation measurements comprising a single, brief (0.5 ms integration time) reading, whereas dose response curves above were derived from summation of signal read continuously over 30 minutes. Vehicle-incubated cells untreated ("–Mesna") or treated (" +MesNa") with MesNA report successful labelling and stripping, respectively, of BG-SS-Lumi4-Tb. Theoretically, no signal should be detected in the +MesNa vehicle condition; that detected may result from non-specific uptake of the Lumi4-Tb probe during labelling, or constitutive internalisation of the GLP-1R. Internalised receptor signal after exendin-phe1 and exendin-phe1-acyl was similar to vehicle treatments, confirming that only a minor degree of agonist-induced internalisation resulted from these compounds.

Rapid recycling was observed after treatment with exendin-phe1 and exendin-phe1-acyl relative to the other agonists (Figure 6.6 B, C). Exendin-phe1-acyl and exendin-asp3-acyl exhibited equal rates of recycling to their unmodified counterparts; exendin-4-acyl, however, achieved a faster rate of recycling than exendin-4, though this difference was not statistically significant. Exendin-4-acyl's increased rate of recycling may – at least in part – underlie the reduced level of net internalisation seen in Figure 6.3, given the aforementioned inability of the DERET assay to discriminate between

receptor internalisation and recycling. This is supported by the lack of difference in internalisation between the two agonists in Figure 6.6 A (though this may be due to differences in assay sensitivity and read time). In summary, as with agonist-induced GLP-1R internalisation, acylation appears to partly affect the recycling properties of the agonists differentially, again with exendin-4 being most affected. Intriguingly, the nature of the trafficking changes caused by acylation of exendin-4 – reduced internalisation and increased recycling – are predictive of enhanced insulinotropism [323].

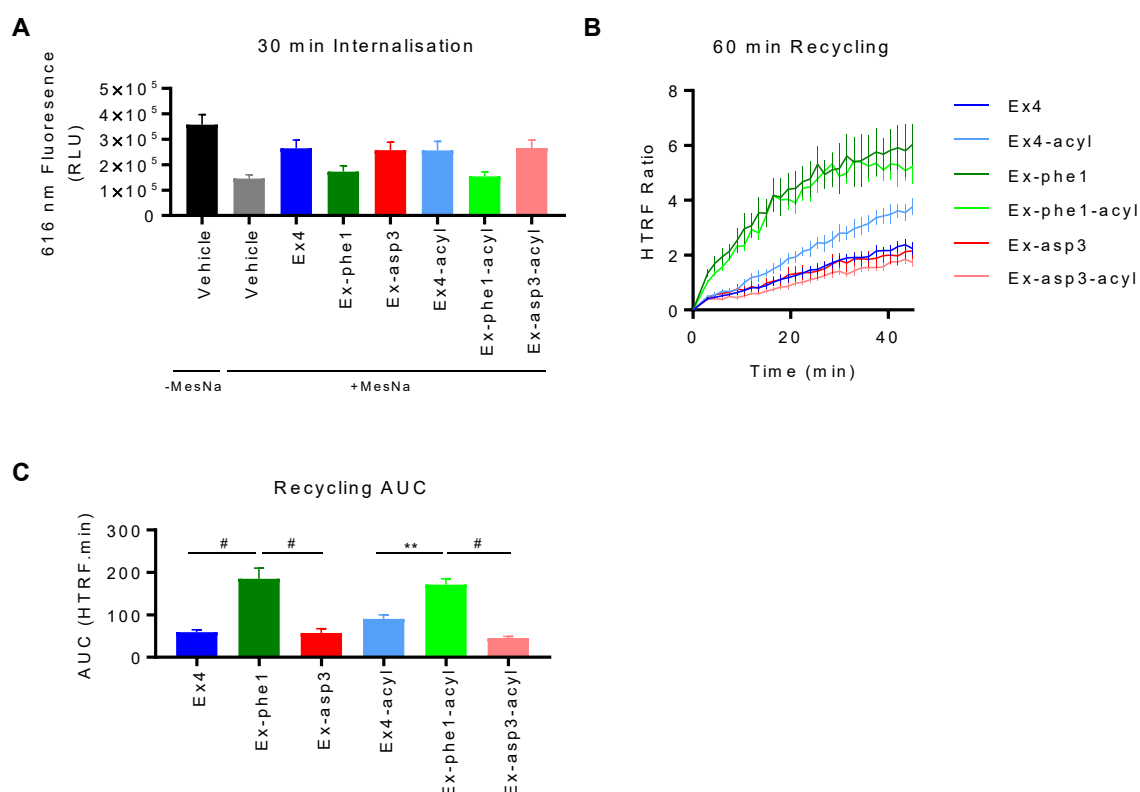


Figure 6.6. GLP-1R recycling in CHO-SNAP-GLP-1R cells.

(A) GLP-1R internalisation after 30 minutes of stimulation with unmodified and acylated GLP-1RAs at 100 nM in CHO-SNAP-GLP-1R cells, $n=5$. Fluorescence reading taken at 616 nm. **(B)** Real-time FRET measurement of LUXendin645 complexed with recycled SNAP-GLP-1R after 30 minutes of agonist stimulation, washout, cleavage of surface SNAP-GLP-1R with MesNa, and treatment with LUXendin645, $n=5$. Signal from plasma membrane-located receptor was measured every 2 minutes for 30 minutes as 665/616 nm signal, and non-specific signal subtracted from readings. **(C)** Area under the curve for data in (B) determined relative to HTRF signal at $t=0$, $n=5$. One-way randomised block ANOVA with Tukey's test. $**p<0.01$, $\#p<0.0001$. Data represented as mean $\pm$ SEM.

6.2.4 Measurement of binding kinetics

The length of time that an agonist remains bound to its receptor – its residence time, defined as the inverse of its dissociation constant ($1/k_{off}$) – has previously been shown to be an important determinant of GLP-1R trafficking [323]. Reduced residence time and thus agonist dissociation within endosomes promotes GLP-1R recycling. Conversely, persistent binding of highly avid agonists within endosomes results in targeting to the degradative pathway. In line with this, pharmacologically increasing the residence time of exendin-4 by application of allosteric modulator 4-(3-(benzyloxy)phenyl)-2-(ethylsulfinyl)-6-(trifluoro-methyl)pyrimidine (BETP) results in reduced GLP-1R recycling without impacting receptor internalisation or signalling potency [323].

To assess whether acylation impacts the binding kinetics of the agonists, and whether changes may be related to the minor alterations in trafficking profiles observed above, competition-based kinetic TR-FRET binding assays were performed. These experiments rely upon FRET between SNAP-GLP-1R labelled with Lumi4-Tb, and FITC-labelled exendin-4 that competes for receptor occupancy with unlabelled forms of the agonists. When unlabelled agonist is added in competition with exendin-4-FITC, the *apparent* rate of association of the latter is altered. Comparison with exendin-4-FITC's binding kinetics when not in competition allows for the association and dissociation rates of the competing unlabelled ligand to be determined, as previously described [544]. Specifically, experiments were conducted with a fixed concentration of exendin-4-FITC in competition with several concentrations of unlabelled agonist. In the assays, endocytosis was pharmacologically inhibited with sodium azide (NaN_3) and 2-deoxyglucose to prevent trafficking-related confounders [545]. For example, changes in the density of cell surface receptors – the rate of which is agonist-dependent – may influence ligand binding and dissociation behaviours. Additionally, FRET signal can be derived from ligand-bound receptor both at the cell surface and within endosomes. However, dissociation dynamics at these two locations may vary due to differences in prevailing ligand concentration and pH. Inhibiting endocytosis therefore allows isolated study of binding kinetics at the plasma membrane in whole cells, as an alternative to the other conventional method using cell membrane preparations. Sequential TR-FRET measurements were taken from the same wells to monitor exendin-4-FITC binding over 60 minutes, with more frequent measurements during the first 20 minutes when changes are most dynamic, but less frequent subsequently to minimise FITC photobleaching.

Results are summarised in Figure 6.7, and Table 6.3 reports the association rate constant (k_{on}), dissociation rate constant (k_{off}), and equilibrium binding constant (K_d - determined as k_{off}/k_{on} and inversely related to receptor affinity) for each of the agonists. Consistent with previous findings, exendin-phe1 exhibited a significantly reduced, and exendin-asp3 a significantly increased residence time (or slower k_{off}) relative to exendin-4. Exendin-phe1 also exhibited a higher k_{on} , yet still a significantly higher K_d (i.e. lower receptor affinity) relative to exendin-4, given larger changes in k_{off} . By contrast, exendin-asp3's decreased k_{off} (longer residence time) was balanced by a decreased k_{on} , thus showing similar receptor affinity to exendin-4.

Acylation resulted in agonist-dependent effects upon binding kinetics. For exendin-4 and exendin-asp3, acylation increased k_{on} but decreased residence time which, depending on the magnitude of respective changes, led to either no overall change (exendin-4 *versus* exendin-4-acyl) or an increase (exendin-asp3 *versus* exendin-asp3-acyl) in receptor affinity (Figure 6.7 C-F). Of note, the residence time of exendin-4-acyl was 2.5-fold shorter than exendin-4, fitting with exendin-4-acyl's increased rate of recycling, reduced levels of receptor internalisation and reduced β -arrestin-2 recruitment. Interestingly though, whilst exendin-asp3-acyl exhibited a significantly longer residence time compared to exendin-4, and a significantly reduced residence time relative to exendin-asp3, all three agonists showed a similar rate of recycling (Figure 6.6). This suggests that, past a certain point, further increases in residence time do not translate into corresponding decreases in recycling rate. The other interesting observation here is that a halving in k_{off} and small increase in k_{on} were seen for exendin-phe1-acyl *versus* exendin-phe1, resulting in a significant increase in receptor affinity for exendin-phe1-acyl. This contrasts with the reduced signalling potency for exendin-phe1-acyl, suggesting that despite improved binding affinity it is less well coupled to intracellular signalling.

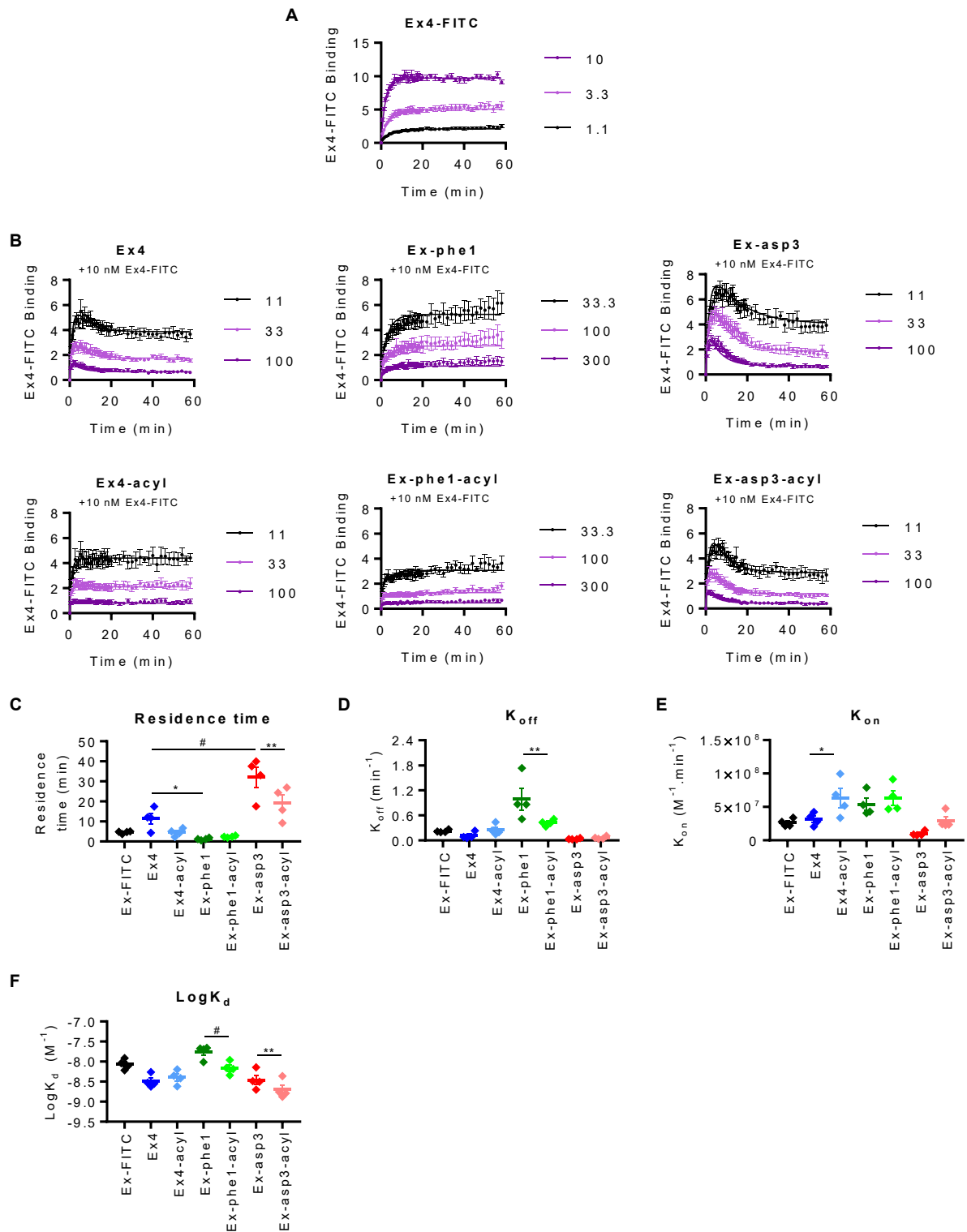


Figure 6.7. Competitive binding kinetics of GLP-1RAs with Ex4-FITC in HEK293-SNAP-GLP-1R cells.

GLP-1R in HEK293-SNAP-GLP-1R cells was labelled with Lumi4-Tb and cells were exposed to agonist after inhibition of endocytosis with NaN₃ and 2-deoxyglucose. Binding was recorded every 40 seconds as 520/620 nm signal, and non-specific signal subtracted from readings. **(A)** Kinetic binding of ex4-FITC at the indicated concentrations, n=4. **(B)** Kinetic binding of 10 nM ex4-FITC in competition with unlabelled GLP-1RAs (unmodified or acylated) at the indicated concentrations, n=4. **(C)** Residence time derived from data in (A) and (B). **(D)** K_{off} derived from data in (A) and (B). **(E)** K_{on} derived from data in (A) and (B). **(F)** LogK_d derived from data in (A) and (B). One-way randomised block ANOVA with Tukey's test. *p<0.05, **p<0.01, #p<0.0001. Data represented as mean ± SEM.

	K_{on} ($M^{-1}.min^{-1}$)	K_{off} (min^{-1})	Residence time (min)	$LogK_d$ (M^{-1})
Ex4-FITC	$2.63 \times 10^7 \pm 2.87 \times 10^6$	0.221 ± 0.016	4.59 ± 0.30	-8.07 ± 0.06
Ex4	$3.16 \times 10^7 \pm 4.79 \times 10^6$	0.115 ± 0.040	11.4 ± 2.70	-8.49 ± 0.08
Ex4-acyl	$6.33 \times 10^7 \pm 1.40 \times 10^7 *$	0.256 ± 0.063	4.56 ± 0.92	-8.4 ± 0.09
Ex-phe1	$5.39 \times 10^7 \pm 8.74 \times 10^6$	0.994 ± 0.260	1.21 ± 0.28	-7.76 ± 0.09
Ex-phe1-acyl	$6.31 \times 10^7 \pm 1.05 \times 10^7$	$0.424 \pm 0.037 **$	2.42 ± 0.22	$-8.16 \pm 0.08 \#$
Ex-asp3	$9.89 \times 10^6 \pm 1.75 \times 10^6$	0.035 ± 0.007	32.0 ± 5.02	-8.46 ± 0.12
Ex-asp3-acyl	$2.96 \times 10^7 \pm 6.06 \times 10^6$	0.062 ± 0.016	$19.1 \pm 4.06 **$	$-8.7 \pm 0.11 **$

Table 6.3. Kinetic binding constants for unmodified and acylated GLP-1RAs.

Values derived from the four independent experiments for which the averaged data is depicted in Figure 6.7. Equilibrium binding constant (K_d) determined as k_{off}/k_{on} . Significance reported in comparison to unmodified peptide e.g. ex4 vs. ex4-acyl. One-way randomised block ANOVA with Tukey's test. * $p < 0.05$, ** $p < 0.01$, # $p < 0.0001$. Data represented as mean $\pm$ SEM.

From the pharmacological evaluation above, it is evident that acylation changes the binding kinetics and signalling properties of these GLP-1RAs. Potential explanations for this include fatty acid chain-mediated interactions with the plasma membrane altering the local concentration of agonist, interactions of the fatty acid chain with the receptor altering the agonist orientation and therefore binding and activation parameters, or changes in peptide secondary structure. The latter has been reported for dual GLP-1R/glucagon receptor (GCGR) agonists, with analogue acylation stabilising the helical secondary structure of the peptide and enhancing potency [546]. For ghrelin, endogenous octanoylation results in increased interaction with the plasma membrane and a consequent increase in local ghrelin concentration [547]. It is posited that this facilitates an increase in ghrelin receptor (GHSR) interaction, and thus underlies the difference in relative potency between unacylated and acylated ghrelin at the GHSR (in the micromolar range for the former and nanomolar range for the latter) [547]. However, which of these effects is at play for the agonists tested here is unclear, though it is tempting to speculate that the acylation-induced increases in receptor affinity (at least for exendin-phe1-acyl and exendin-asp3 acyl) reflect constructive interactions of the fatty acid with the receptor or membrane.

6.2.5 Measurement of insulin secretion *in vitro*

To examine the potential impacts of acylation-induced signalling and trafficking alterations upon functional responses to the agonists, insulin secretion assays were performed. Assays conducted thus far were in cell lines expressing the human GLP-1R, whereas rat pancreatic β -cell line INS-1 832/3 was used for insulin secretion assays. cAMP assays were therefore conducted in INS-1 832/3 to rule out the existence of species-specific signalling responses to the acylated agonists. Moreover, the impact of variable concentrations of foetal bovine serum (FBS) in the cAMP assay media was tested to ascertain an appropriate concentration for use in insulin secretion assays. The inclusion of FBS in insulin secretion assays is to better mimic *in vivo* dynamics: in the circulation, acylated peptide must dissociate from serum albumin in order to activate the GLP-1R, which likely impacts signalling responses.

cAMP accumulation responses were largely matched between the human and rat GLP-1R (compare Figure 6.8 A-C with Figure 6.1 A and Table 6.1) similar values for potency and efficacy. The only exception was exendin-phe1-acyl's reduced efficacy at the rat GLP-1R, making it a partial agonist. With regard to the effect of FBS, its addition impacted agonist potency (but not efficacy), but no difference was observed between 1% and 10% FBS conditions (Figure 6.8 B, C). The reduced potency of agonists in the absence of FBS is likely due to peptide loss from binding to plasticware. Given the lack of difference between 1% and 10% conditions, insulin secretion assays were conducted with 1% FBS.

The expected pattern of insulin release was observed with non-modified agonists, with exendin-phe1 being the most efficacious and exendin-asp3 the least, as previously reported [323]. Again, acylation impacted insulin secretion responses in an agonist-dependent manner (Figure 6.8 D, E, Table 6.4). Acylation resulted in increased maximal insulin secretion for exendin-4 and exendin-asp3, but decreased efficacy for exendin-phe1 such that exendin-phe1-acyl no longer outperformed the other acylated agonists.

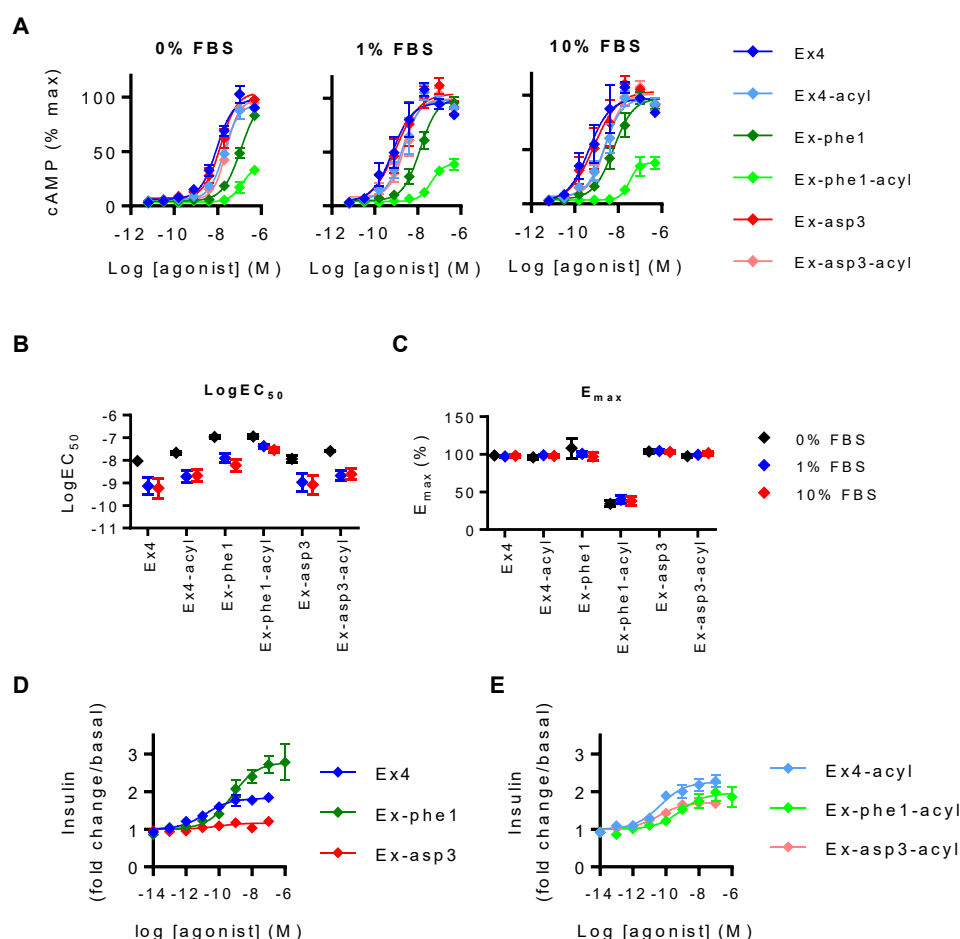


Figure 6.8. cAMP accumulation and insulin secretion responses to unmodified and acylated GLP-1RAs in INS-1 832/3 cells.

(A) cAMP responses to unmodified and acylated forms of exendin-4, exendin-phe1 and exendin-asp3 in INS-1 832/3 cells with either 0%, 1% or 10% FBS in stimulation medium, 10 min incubation. Data are expressed as a percentage of the maximal response in the assay with four-parameter logistic fit of data, $n=3$. (B) LogEC₅₀ values for the data in (A). (C) E_{max} values for the data in (A). (D-E) Insulin secretion responses of unmodified and acylated GLP-1RAs in INS-1 832/3 cells with 1% FBS in the stimulation medium, 16 hour incubation, $n=6$. Four parameter logistic fit of the data shown. Data are represented as mean $\pm$ SEM.

Peptide	Insulin Secretion				
	pEC ₅₀ (nM)	ΔlogEC ₅₀	EC ₅₀ Ratio	E _{max} (fold change/basal)	Ratio
Ex4	10.96 ± 0.18			1.78 ± 0.10	
Ex4-acyl	10.6 ± 0.14	0.37 ± 0.05	2.31	2.17 ± 0.16 *	1.23
Ex-phe1	9.38 ± 0.21			2.67 ± 0.21	
Ex-phe1-acyl	9.20 ± 0.16	0.19 ± 0.12	1.54	1.95 ± 0.20 #	0.73
Ex-asp3	9.51 ± 0.41			1.17 ± 0.09	
Ex-asp3-acyl	10.6 ± 0.26 *	-1.10 ± 0.20	0.08	1.69 ± 0.11 **	1.44

Table 6.4. Potency and efficacy for unmodified and acylated GLP-1RAs for insulin secretion in INS-1 832/3 cells.

Values are derived from data in Figure 6.8. “ΔlogEC₅₀” is the difference in logEC₅₀ values between unmodified and acylated counterparts. “EC₅₀ Ratio” describes the ratio between the EC₅₀ values for unmodified and acylated counterparts. “Ratio” denotes E_{max} value relative to that of the equivalent unmodified compound. E_{max} values normalised to global maximum in individual experiments. Significance reported in comparison to unmodified peptide e.g. ex4 vs. ex4-acyl. One-way randomised block ANOVA with Tukey’s test. *p<0.05, **p<0.01, #p<0.0001. Data reported as mean ± SEM from 6 independent experiments.

Relating the results from insulin secretion assays to those from trafficking and binding kinetics experiments, a consistent story emerges for exendin-4 *versus* exendin-4-acyl (Table 6.5). Relative to exendin-4, exendin-4-acyl had a reduced receptor residence time, reduced β-arrestin recruitment, reduced internalisation, an increased recycling rate, matched receptor potency, and enhanced insulinotropism *in vitro*. As previously reported [323], the likely effect of this trafficking phenotype is increased levels of plasma membrane-located, sensitised GLP-1R, leading to prolonged signalling and insulin secretory responses. By contrast, acylation did not confer benefits to exendin-phe1-acyl. Despite having a similar trafficking phenotype to exendin-phe1, exendin-phe1-acyl’s loss of potency and efficacy (the latter albeit only at the rat GLP-1R) likely underlies its poor insulinotropism *in vitro*.

Finally, exendin-asp3-acyl presents a more confusing *in vitro* phenotype. Its reduced residence time and β-arrestin recruitment relative to exendin-asp3 did not translate into changes in receptor trafficking (internalisation and recycling are largely matched for the two agonists). Potency and efficacy at both the rat and human GLP-1R were also equal. Despite this, exendin-asp3-acyl had significantly higher potency and efficacy for insulin secretion *in vitro*. This set of observations may potentially be reconciled by the fact that the role for β-arrestins in GLP-1R desensitisation may supersede their role in GLP-1R trafficking, as has been previously suggested [323]. Supporting this, the effect of dual β-arrestin knockdown only has modest effects upon GLP-1R endocytosis whilst consistent increases in insulin secretion are observed. This discrepancy suggests a desensitisation-related role for β-arrestins in negatively modulating insulin secretion in pancreatic β-cells that is independent of their effects upon trafficking.

Overall, the conducted *in vitro* studies highlight that the effect of acylation of GLP-1RAs upon signalling, trafficking, binding kinetics and functional responses shows some consistent impacts of acylation on all agonists, but also areas of variation. How these changes translate into effects *in vivo* is of interest.

Peptide	Bias	cAMP	β -arrestin-2	Internalisation	Recycling	Residence time	K_{on}	Binding Affinity	Insulin
Ex4	N	++	++	++	+	++	+	++	++
Ex4-acyl	N/G	++	+	+(+)	++	+(+)	++	++	++(+)
Ex-phe1	G	+	-	-	+++	-	++	+	+++
Ex-phe1-acyl	G	+	-	-	+++	-	++	+(+)	++
Ex-asp3	β	++	+++	++	+	++++	-	++	+
Ex-asp3-acyl	β	++	+	++	+	+++	+	+++	++

Table 6.5. Summary of the *in vitro* properties of unmodified and acylated GLP-1RAs.

For bias, “N” denotes neutral agonist, “G” denotes G-protein-biased agonist, and “ β ” denotes β -arrestin-biased agonist. Agonist performance for each of the assays are expressed relative to one another on a crude scale of “-” as 0 to “++++” as +4; (+) denotes an increment of +0.5. Magnitude comparisons are only relevant within columns.

6.2.6 Anorectic effects of acylated GLP-1RAs in lean mice

As observed above, large differences in signalling potency exist between exendin-4-acyl and exendin-phe1-acyl (EC_{50} ratio of 18.4 for cAMP accumulation at human GLP-1R), complicating “fair” comparisons of their performance *in vivo* (from the point of view of determining the impact of bias, even if not from a therapeutic perspective). Performing agonist dose matching for *in vivo* studies based solely upon *in vitro* EC_{50} values would be erroneous given the vastly increased complexity of drug responses in an entire organism as compared to an immortalised cell line. Given that the dose-limiting factor for GLP-1RAs is the side effect of nausea, doses that were “equiactive” for acute (1 hour) food intake reduction were identified and used as a proxy for nausea and therefore tolerability, and as a marker of biological potency *in vivo*. Anorectic responses to exendin-4-acyl, exendin-phe1-acyl and exendin-asp3-acyl at 2, 10 and 50 nmol/kg were assessed in lean mice over 72 hours (Figure 6.9).

Cumulative food intake for all doses over the first 8 hours is shown in Figure 6.9 A and separately for each dose in Figure 6.9 C, with selected intervals highlighted in Figure 6.9 B, D-F. As anticipated, given its lower potency *in vitro*, exendin-phe1-acyl had weaker anorectic effects over the first 24 hours compared to exendin-4 at doses of 2 and 10 nmol/kg. Interestingly, exendin-asp3-acyl was also less effective than exendin-4-acyl despite similar potency *in vitro* (EC_{50} ratio of 1.60 for cAMP accumulation at human GLP-1R), giving credence to the notion that *in vitro* potency can be a poor correlate of *in vivo* responses. When compared to the subtle differences between anorectic effects of non-acylated exendin-asp3 and exendin-4 reported in Chapter 3, exendin-asp3-acyl’s weaker effects relative to exendin-4-acyl could be explained by increased GLP-1R desensitisation as a result of the combined impact of increased absolute level of β -arrestin-2 recruitment relative to exendin-4-acyl (Table 6.1) and its improved pharmacokinetics leading to higher, and sustained concentrations of agonist at CNS appetite regulating nuclei, thereby allowing the effects of its enhanced β -arrestin-2 recruitment to be manifested. The latter phenomenon mimics (though to a lesser extent) the effects of direct ICV delivery of exendin-asp3 in Chapter 5 (Figure 5.6). It is unclear whether this can explain the

reduced anorectic efficacy of exendin-asp3-acyl acutely at 1 hour, but is compatible with the fact that this difference continues to widen over subsequent hours.

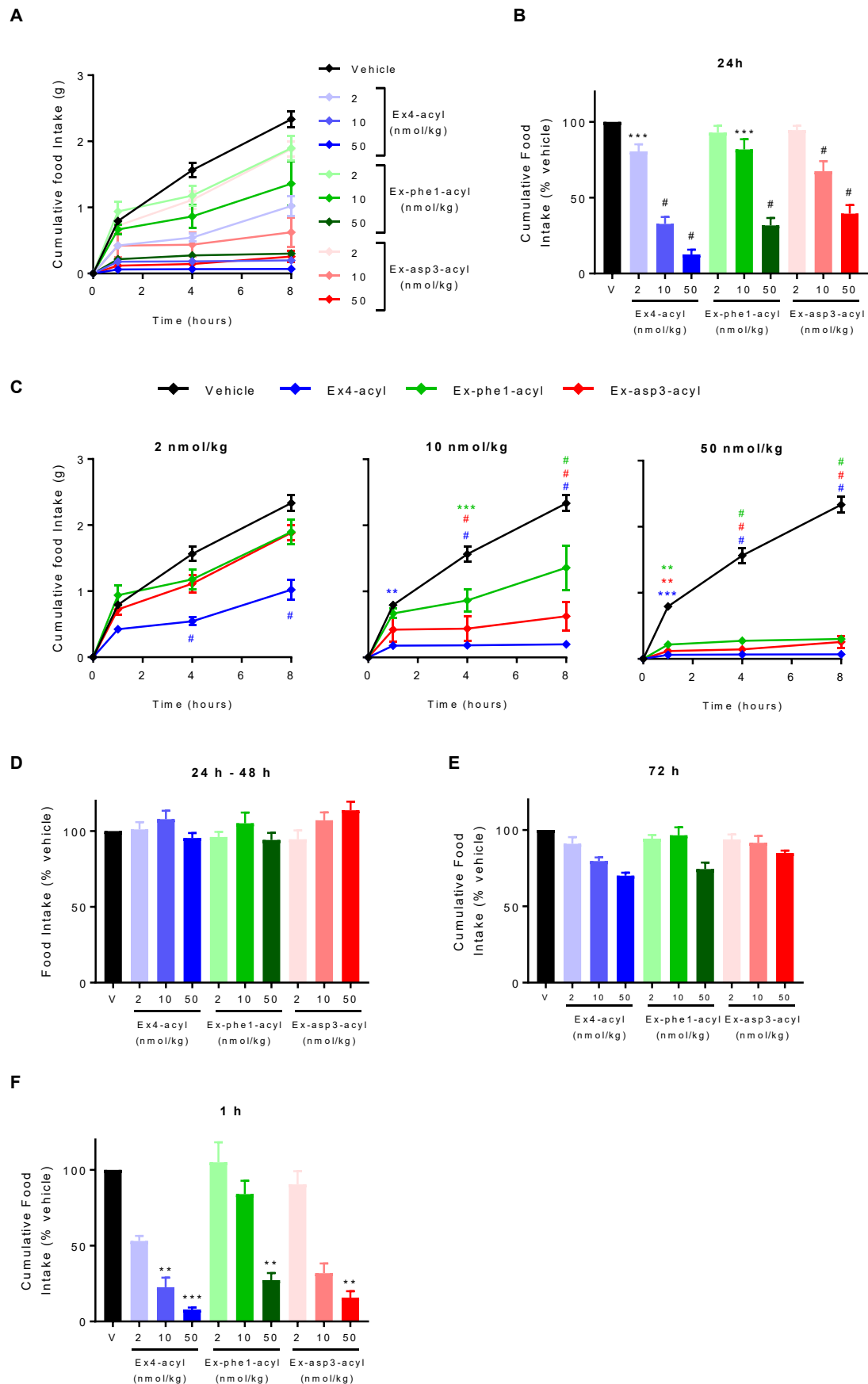


Figure 6.9. Anorectic effects of acylated GLP-1RAs in lean mice.

(A) Cumulative food intake over the first 8 hours in lean, male C57Bl/6 mice after intraperitoneal administration of ex4-acyl, ex-phe1-acyl or ex-asp3-acyl at the indicated doses, n=8. (B) As in (A) but at 24 hours. (C) Data in (A) but separated into tested doses. (D) As in (A) but food intake between 24 and 48 hour time points. (E) As in (A) but at 72 hours. (F) As in (A) but at 1 hour. Two-way ANOVA with Dunnett's test, with significance vs. saline. *p<0.05, **p<0.01, ***p<0.001, #p<0.0001. Data represented as mean $\pm$ SEM.

At the highest dose of 50 nmol/kg, all agonists induced comparable reductions in food intake over the first 8 hours, suggesting that doses increases can overcome differences in potency. Accordingly, at 24 hours when bioavailability would have decreased due to renal clearance, differences re-emerged with exendin-4 again most effective (Figure 6.9 B). Between the 24- and 48-hour marks, the anorectic effects of the acylated agonists had worn off for all doses, or were at least compensated for by counterregulatory mechanisms (Figure 6.9 D). As anticipated, this represents a prolongation of anorectic effects relative to the non-acylated forms, the anorectic effects of which at equal doses cease between the 8- and 24-hour time points. At 72 hours, cumulative food intake for high doses of the agonists remained reduced relative to vehicle-treated animals (Figure 6.9 E), suggesting that any compensatory hyperphagia at later time points did not negate the overall effects of agonist-induced hypophagia in the first 24 hours.

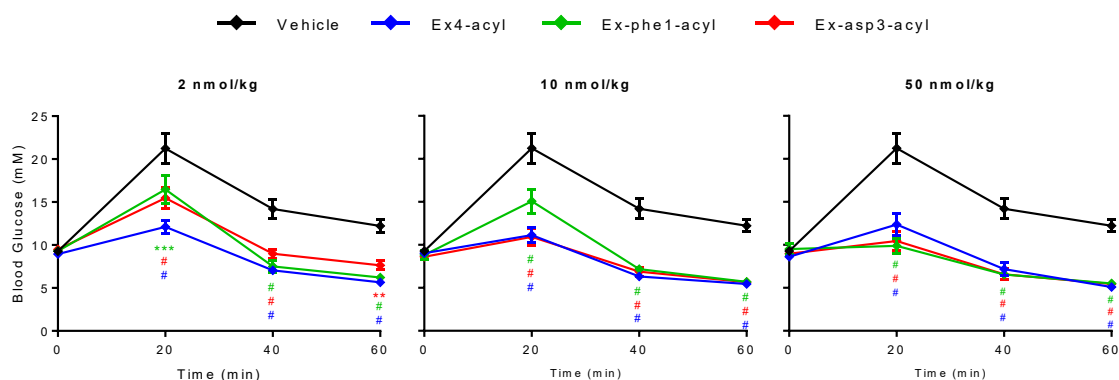
As aforementioned, one aim of this study was to identify “equiactive” doses on the basis of nausea for subsequent comparison in GTTs. With exendin-4-acyl at 2 nmol/kg as a reference dose (food intake of 53% of vehicle), a dose of 10 nmol/kg for both exendin-phe1-acyl (83% of vehicle) and exendin-asp3-acyl (31% of vehicle) were identified as approximately matched (Figure 6.9 F). Admittedly, this dose selection appears to provide an “advantage” for exendin-asp3-acyl and “disadvantage” exendin-phe1-acyl, if using the degree of food intake suppression as a marker of biological activity. An alternative pairing would have been exendin-phe1-acyl at 50 nmol/kg (food intake 27% of vehicle), exendin-4-acyl at 10 nmol/kg (22% of vehicle) and exendin-asp3-acyl at 50 nmol/kg (15% of vehicle), but the profound effect on food intake reduction with these doses was considered to be excessive.

6.2.7 Glycaemic responses to acylated GLP-1RAs in lean mice

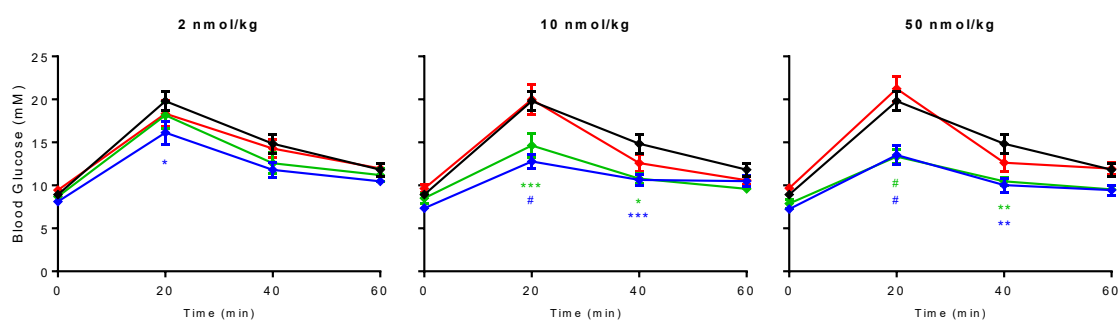
To investigate the glucoregulatory effects of the acylated agonists, IPGTTs were conducted acutely and 72 hours after peptide administration at 2, 10 and 50 nmol/kg. The 72-hour delayed GTT was intended as the equivalent of the 6-hour delayed GTT for non-acylated agonists, accounting for the anticipated extended half-life of the acylated agonists. In acute GTTs, all agonists at all doses had significant anti-hyperglycaemic effects (Figure 6.10 A, C). Exendin-phe1-acyl had weaker effects at 2 and 10 nmol/kg doses, in keeping with its reduced potency and insulinotropism *in vitro*. As with non-acylated peptides, time-dependent bias differences emerged at 72 hours: β -arrestin-biased exendin-asp3-acyl no longer improved glucose handling relative to vehicle, whilst exendin-4-acyl and exendin-phe1-acyl continued to cause glycaemic improvements (Figure 6.10 B, D). Effects were minimal at 2 nmol/kg, likely due to renal clearance of the peptides. Unlike non-acylated peptides, exendin-phe1-acyl did not outperform dose-equal exendin-4-acyl after a delay; again, this is likely attributable to exendin-phe1-acyl's reduced potency, as well as the fact that that exendin-4-acyl recruited less β -arrestin, showed a favourable trafficking profile, and was more insulinotropic relative to exendin-4. When comparing “equiactive” doses identified above to try to correct for differences in potency, 10 nmol/kg exendin-phe1-acyl performed marginally, though not significantly better than 2 nmol/kg

exendin-4-acyl (Figure 6.10 E). In addition to the differences in their action at the GLP-1R described in the *in vitro* sections, this may also reflect the imperfect nature of the dose matching, as 10 nmol/kg exendin-phe1-acyl was still less acutely anorectic as 2 nmol/kg exendin-4-acyl. By 7 days, the anti-hyperglycaemic effects of all agonists had worn off (Figure 6.10 F).

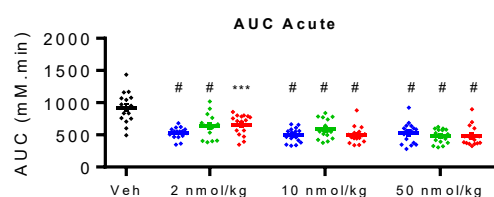
A Acute



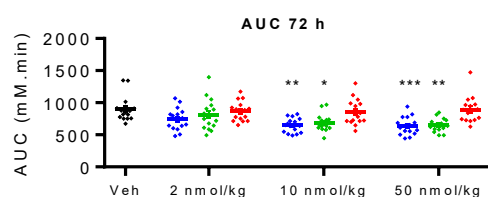
B 72 hours



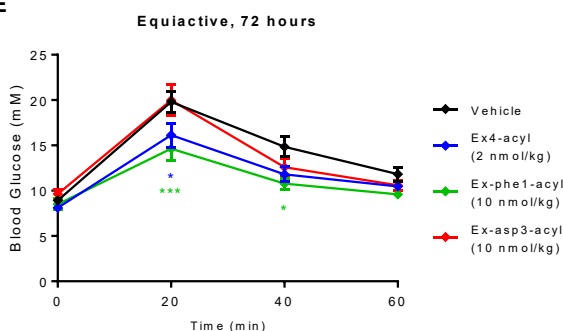
C



D



E



F

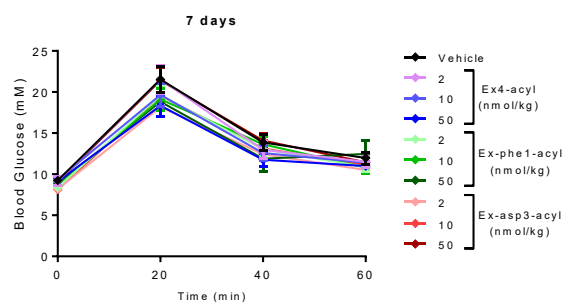


Figure 6.10. Glycaemic responses after administration of acylated GLP-1RAs in lean mice.

(A) Blood glucose during an IPGTT (2 g/kg glucose) in lean, male C57Bl/6 mice after intraperitoneal agonist injection at the indicated dose, n=16/group. **(B)** As for (A) but with a 72 hour delay between agonist administration and glucose challenge, n=16/group. **(C)** Area under the curve for (A), determined relative to glucose at t=0. **(D)** Area under the curve for (B), determined relative to glucose at t=0. **(E)** Selected data from (C), comparing “equiactive” doses of the acylated agonists. **(F)** As for (A) but with 7 days between agonist administration and glucose challenge, n=16/group. Two-way ANOVA with Dunnett’s test for (A) and (B), or one-way ANOVA with Tukey’s test for (C) and (D), with significance vs. saline for all tests. *p<0.05, **p<0.01, ***p<0.001, #p<0.0001. Data represented as mean ± SEM.

Overall, acylation considerably extended the anti-hyperglycaemic effects of exendin-4 and exendin-phe1. It is interesting to note that agonist-induced effects upon pancreatic β -cells were clearly still evident at 72 hours, whereas their effects upon food intake cease between 24 and 48 hours post administration. There are a few potential explanations for this discrepancy in effect duration. Firstly, compensatory changes may occur to correct the energy deficit as a result of hypophagia in the first 24 hours, thereby nullifying the anorectic effects of remaining agonist in the system. Secondly, as reported in Chapter 5, the EC_{50} for GLP-1R-coupled cAMP accumulation was over 100-fold higher in neurons than β -cells (Table 5.1); this tissue difference in agonist potency would mean that, with progressive renal clearance of the peptides, the sub-anorectic systemic concentration would be reached before the sub-insulinotropic concentration. Alternatively put, plasma levels of agonist at 72 hours may be high enough for significant β -cell, but not neuronal GLP-1R activation. Thirdly, there may be differences in the ability of the acylated compounds to access the brain *versus* the pancreas. Binding to albumin may reduce the free fraction of peptide capable of crossing the BBB/BCSFB, thereby reducing brain entry. Increasing the hydrophobicity of substances may also – contrary to classical thinking - lead to decreased BBB penetration due to increased uptake by peripheral tissues, trapping in the plasma membranes of BBB endothelial cells, or increasing the likelihood of it becoming a substrate for BBB efflux pumps [421].

6.2.8 Glycaemic responses to acylated GLP-1RAs in DIO mice

Conducting studies in lean mice provides a convenient and cost-effective method for the initial screening of compounds and doses. However, ultimately, responses in rodent models of T2DM act as a better predictor of clinical efficacy. Acute and 72-hour delayed GTTs were therefore conducted in mice fed high-fat diet for 3 months to cause diet-induced obesity (DIO) and glucose intolerance. The “equiactive” doses identified above (2 nmol/kg for exendin-4-acyl, 10 nmol/kg for exendin-phe1-acyl and exendin-asp3-acyl) were used for these studies. All agonists had significant anti-hyperglycaemic effects in the acute study (Figure 6.11 A, B). Note that effects appear more pronounced than in lean mice; this is at least in part due to an experimental error in which agonist was administered two hours prior to the GTT, hence the lower blood glucose for agonist-treated mice compared to saline at time “0”. Importantly, blood glucose readings at time “-120” demonstrated equal basal blood glucose between the groups (due to weight-matching), excluding the possibility that differential glucoregulatory responses were due to differences in the severity of diabetic syndrome between the groups. At 72 hours, exendin-asp3-acyl failed to improve glucose handling, whereas exendin-4-acyl and exendin-phe1-acyl caused significant anti-hyperglycaemic effects (Figure 6.11 C, D). G-protein-biased exendin-phe1-acyl significantly outperformed β -arrestin-biased exendin-asp3-acyl, and a trend towards significance (p=0.056) in AUC was observed between exendin-phe1-acyl and exendin-4-acyl.

Notably, a single mouse in the exendin-phe1-acyl cohort prevented attainment of significance *versus* exendin-4-acyl; without this mouse, $p=0.0171$.

It is intriguing to note exendin-phe1-acyl's improved effect upon a diabetic background. Unlike in DIO mice, the difference in exendin-phe1-acyl and exendin-4-acyl's anti-hyperglycaemic effects in lean mice did not come close to statistical significance. This difference may be attributable to changes in β -cell functionality in the hyperglycaemic and dyslipidaemic diabetic state. Indeed, chronic hyperglycaemia results in GLP-1R downregulation in pancreatic β -cells [548], lipotoxicity disrupts islet connectivity [155], and dyslipidaemia leads to changes in β -cell membrane lipid composition [549]. Providing a direct link to the biased agonists tested here is recent work from the laboratory evidencing the membrane lipid microenvironment to control GLP-1R responses to exendin-4, exendin-phe1 and exendin-asp3 differentially [532]. Thus, pathological changes in β -cell membrane composition and other properties may underlie the agonist-specific changes observed here in lean *versus* DIO mice.

The data also evidence the importance of *in vivo* testing in a physiologically-relevant model, as both prolonged incubation insulin secretion assays *in vitro* and delayed GTTs in lean mice did not predict exendin-phe1-acyl's improved effect relative to exendin-4-acyl. In summary, exendin-4-acyl and exendin-phe1-acyl cause significant improvements in glucoregulation over 72 hours in a rodent model of T2DM, posing them as therapeutically favourable given the potential for reduced frequency of dosing.

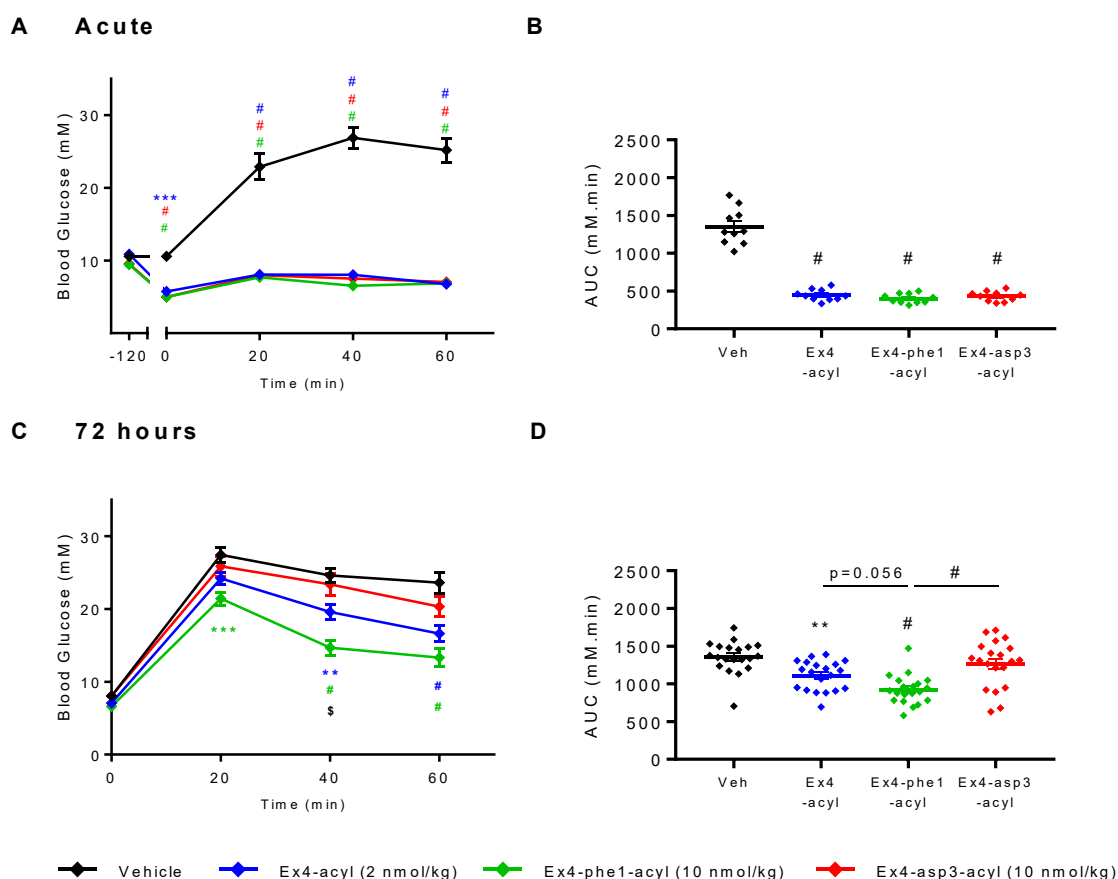


Figure 6.11. Glycaemic responses after administration of acylated GLP-1RAs in DIO mice.

(A) Blood glucose during IPGTT (2 g/kg glucose) in DIO, male C57Bl/6 mice after intraperitoneal agonist injection at the indicated dose, n=10/group. (B) Area under the curve for (A), determined relative to glucose at t=0. (C) and (D) As for (A) and (B) but with a 72 hour delay between agonist administration and glucose challenge, n=20/group. Two-way ANOVA with Tukey's test for (A) and (C), with significance vs. saline indicated, except for \$ sign in (C) that denotes significance between ex4-acyl and ex-phe1-acyl. One-way ANOVA with Tukey's test for (B) and (D) with significance vs. saline unless denoted by lines between groups. **p<0.01, ***p<0.001, #p<0.0001. Data represented as mean $\pm$ SEM.

6.2.9 Anorectic effects of acylated GLP-1RAs in DIO mice

Given that the weight-lowering effects of long-acting GLP-1RAs largely reflect reductions in food intake (with minimal effects upon energy expenditure) [550], [551], the anorectic effects of the acylated agonists were assessed in DIO mice (Figure 6.12). Food intake reduction over the first hour – used as a proxy for nausea – was matched between the treatments, suggesting the achievement of equiactive doses based upon this therapeutically relevant parameter (Figure 6.12 A). Significant and similarly potent anorectic effects were observed over the first 8 hours for all three agonists (Figure 6.12 B). However, at 24 hours, exendin-phe1-acyl-treated mice showed significantly lower cumulative food intake compared to those treated with exendin-4-acyl, suggesting sustained anorectic action of the former (Figure 6.12 C). Food intake between 24 and 48 hours also suggested exendin-phe1-acyl to have sustained anorectic effects relative to exendin-4-acyl, though differences were not significant (Figure 6.12 D). Weight loss data reflected the anorectic effects of the agonists, with exendin-phe1-acyl resulting in greater – though not statistically significant (p=0.111) - weight loss at 24 hours compared to exendin-4-acyl (Figure 6.12 F, G). Importantly, differences in absolute body weight or body weight loss were not significant at 72 hours, suggesting that the improved anti-hyperglycaemic effects of exendin-phe1-acyl *versus* exendin-4-acyl seen in DIO mice above were not solely due to differences in weight loss. As in lean mice, cumulative food intake at 72 hours was still lower than that of vehicle-treated mice (Figure 6.12 E), indicating that the overall effects of earlier hypophagia and consequent weight loss (Figure 6.12 G) were not nullified by any compensatory hyperphagia occurring at later time points.

Thus, in line with its prolonged glucoregulatory effects in DIO mice, exendin-phe1-acyl exhibited subtly stronger anorectic efficacy than both exendin-4-acyl and exendin-asp3-acyl over 24 hours. Importantly, taking the first hour suppression of food intake as a surrogate of nausea, this was not accompanied by commensurate loss in tolerability. Exendin-phe1-acyl's improved anorectic efficacy in DIO *versus* lean mice provides further support for pathological changes associated with obesity and T2DM leading to agonist-specific signalling changes. It has been demonstrated that exendin-4-mediated hypophagia is attenuated in obese rodents [552], [553], and that the GLP-1R expression is reduced in hypothalami from T2DM patients [554]; however, the mechanistic link between pathological changes such as these and alterations in biased GLP-1R agonism are yet to be determined.

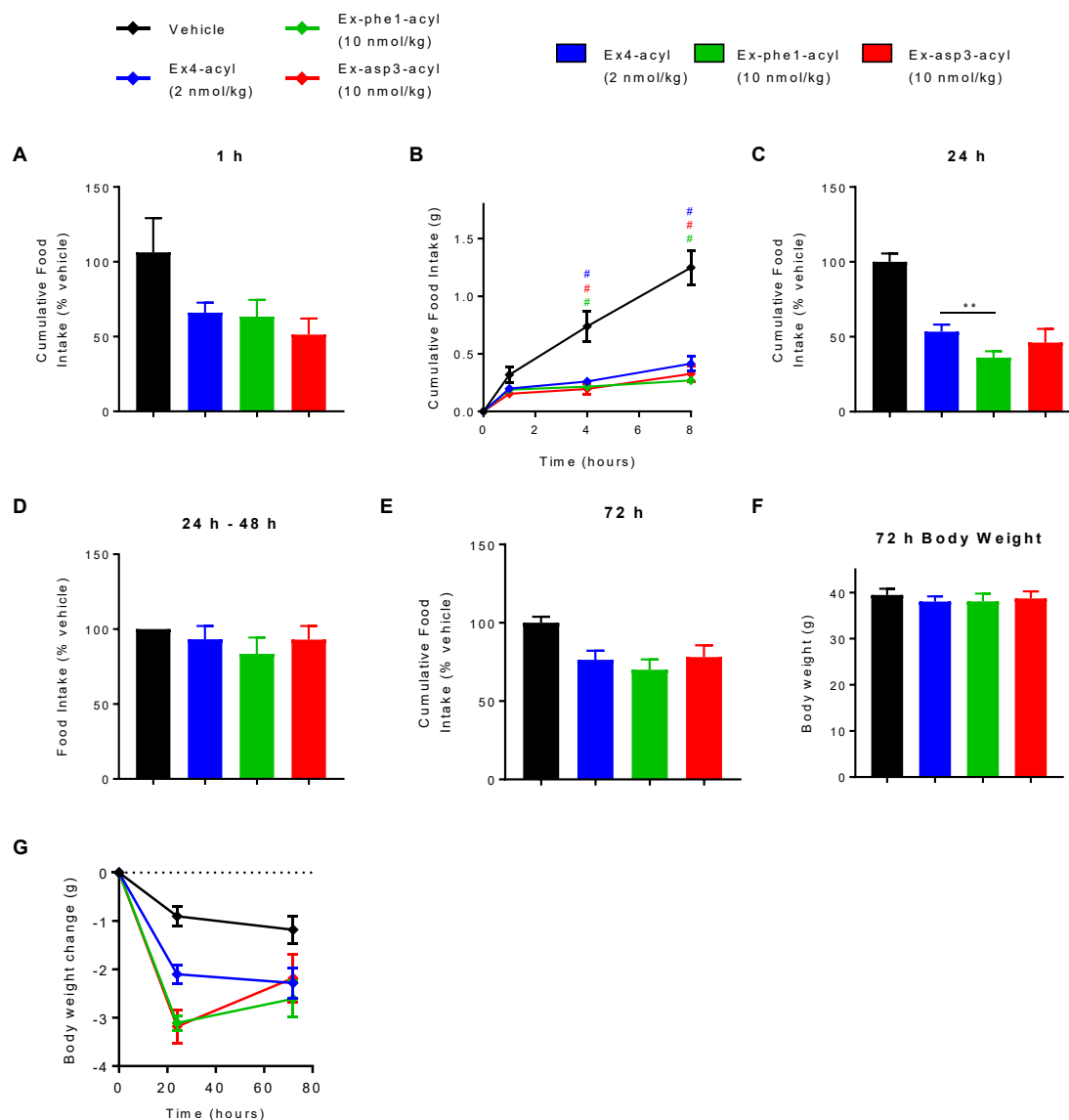


Figure 6.12. Anorectic effects of acylated GLP-1RAs in DIO mice.

(A) Food intake over the first hour in DIO, male C57Bl/6 mice after intraperitoneal administration of ex4-acyl, ex-phe1-acyl or ex-asp3-acyl at the indicated doses, $n=10/\text{group}$. (B) Cumulative food intake over the first 8 hours in mice in (A). Two-way ANOVA with Tukey's test, with significance vs. saline. (C) Cumulative food intake for mice at 24 hours. Two-way ANOVA with Tukey's test. (D) Food intake for mice in (A) between 24 and 48 hour time points. (E) Cumulative food intake for mice at 72 hours. (F) Absolute body weight of mice in (A) at 72 hour time point. (G) Body weight change for mice in (A). ** $p<0.01$, # $p<0.0001$. Data represented as mean $\pm$ SEM.

6.2.10 Measurement of plasma levels of acylated GLP-1RAs

To investigate whether pharmacokinetic differences contributed to the differences observed in physiological testing, lean mice were administered 100 nmol/kg of exendin-4-acyl, exendin-phe1-acyl or exendin-asp3-acyl, and blood collected 24 and 72 hours later. As before, plasma levels were measured by an ELISA that recognises the C-terminus of exendin-4. Peptide recovery was assessed and found to be equal for the acylated peptides, though around 30% of non-acylated exendin-4

(Figure 6.13 A). This may reflect steric hindrance of antibody binding caused by the fatty acid chain. Whilst at 24 hours there were no differences between plasma levels of the acylated agonists, by 72 hours exendin-phe1-acyl was present at significantly higher levels (Figure 6.13 B, C). Exendin-phe1-acyl's superior anti-hyperglycaemic effect at 72 hours could therefore be due, at least in part, to elevated plasma levels relative to the other two acylated agonists.

The extended half-life of exendin-phe1-acyl may potentially be explained by the phenomenon of target-mediated drug disposition (TMDD): where a drug is bound to its pharmacologic target site with high affinity and to a significant extent such that this interaction affects the pharmacokinetic characteristics of the drug [555]. One process that contributes to TMDD of peptide therapeutics is receptor-mediated endocytosis (RME), with ligands that have a higher k_{on} , lower k_{off} , higher receptor-ligand internalisation rate, and lower receptor-ligand recycling rate resulting in greater clearance via RME [556]. Supporting a role for TMDD specifically for GLP-1R ligands is the fact that – despite renal clearance being the main mechanism of exendin-4 elimination [238] – exendin-4 is still cleared in bilaterally nephrectomised rats, albeit more slowly [557]. Moreover, TMDD with RME parameters has been successfully modelled for exendin-4 in rats, monkeys and humans, and diabetic rats, with correspondence between theoretical modelling and *in vivo* observations [558], [559]. Thus, exendin-phe1-acyl, which induces lower levels of receptor internalisation, higher rates of receptor recycling, and has a higher k_{off} may show reduced clearance via this mechanism, leading to its extended pharmacokinetic profile. The lack of difference in plasma levels at 24 hour supports the fact that TMDD plays a subsidiary role relative to glomerular filtration in the clearance of GLP-1RAs; more time is required for the likely subtle differences in TMDD rate between the agonists to be manifested. More detailed pharmacokinetic studies would need to be performed to establish whether this speculative mechanism indeed applies to the acylated biased GLP-1RAs described here.

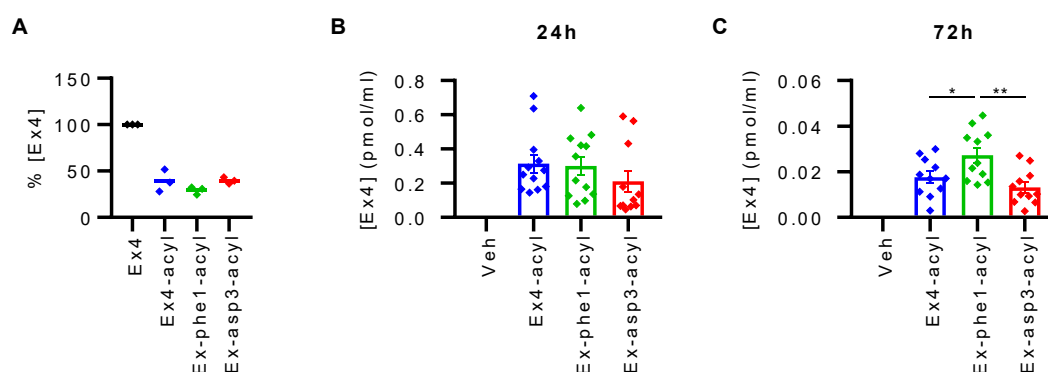


Figure 6.13. Measurement of plasma levels of acylated GLP-1RAs.

(A) Measured exendin-4 levels in ELISA buffer spiked with exendin-4, exendin-4-acyl, exendin-phe1-acyl or exendin-asp3-acyl, and expressed after normalisation to exendin-4 values, $n=3$. **(B)** Plasma exendin-4 concentration 24 hours after intraperitoneal administration of 100 nmol/kg of exendin-4-acyl, exendin-phe1-acyl or exendin-asp3-acyl, $n=7-8$ for vehicle, $n=11-12$ /group for treatments. **(C)** As in (B) but at 72 hours. One-way ANOVA with Tukey's test. * $p<0.05$, ** $p<0.01$. Data represented as mean $\pm$ SEM.

In addition, a study was conducted directly comparing plasma levels of exendin-4 and exendin-4-acyl at 1, 24 and 72 hours post intraperitoneal injection at 100 nmol/kg in mice. Plasma levels of exendin-

4-acyl were found to be 1.75-fold, 15 872-fold, and 86-fold higher than exendin-4 at the 1, 24 and 72 hour time points, respectively (Figure 6.14; note log scale for y-axis). The slightly higher concentration of the acyl ligand at the 1 hour time-point is compatible with the short half-life of exendin-4 (20-30 minutes in mice [444]). Significantly higher relative levels of exendin-4-acyl at the later time points confirm the anticipated effect of acylation-induced half-life protraction, presumably due to the mechanisms of self-aggregation and plasma albumin binding discussed in section 6.1.1.

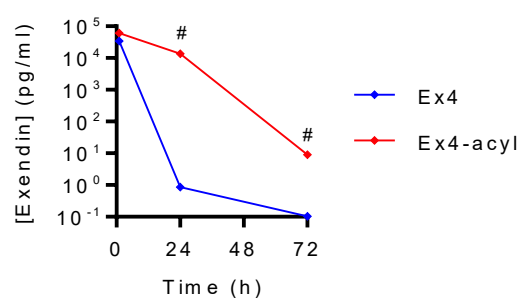


Figure 6.14. Measurement of plasma levels of exendin-4 and exendin-4-acyl.

Plasma exendin-4 concentration at 1, 24 and 72 hours after intraperitoneal administration of 100 nmol/kg of exendin-4 or exendin-4-acyl, n=8. In-assay correction applied for differences in ELISA peptide recovery (see Figure 6.13). Repeated measures two-way ANOVA with Tukey's test. #p<0.0001. Data represented as mean ± SEM.

6.3 Discussion

The work of this chapter concerned the *in vitro* and *in vivo* characterisation of acylated, and therefore pharmacokinetically improved forms of exendin-4, exendin-phe1, and exendin-asp3. The foci of the following discussion are threefold:

1. The effects of acylation upon the *in vitro* properties of the biased GLP-1RAs;
2. The properties of the acylated agonists *in vivo* and how these relate to their *in vitro* characteristics; and
3. Differences in the effects of the acylated agonists in the physiological *versus* pathological obese, diabetic state.

6.3.1 *In vitro* properties of acylated biased GLP-1RAs

As summarised in Section 6.2.5, acylation had variable effects upon the binding kinetics, signalling properties, trafficking profiles, and insulinotropic efficacy of the biased GLP-1RAs. Given the use of identical conjugation moiety (C16) and position for acylation (Lys40), these results highlight the impact of the peptide backbone upon the resultant effects of acylation, with even single amino acid differences altering acylation-induced changes to receptor binding and downstream events.

- For exendin-4, acylation was advantageous, with exendin-4-acyl exhibiting a favourable *in vitro* profile akin to “typical” G-protein biased GLP-1RAs: reduced receptor residence time, β -arrestin recruitment, agonist-induced internalisation, and an increased rate of GLP-1R recycling. As predicted, commensurate increases in insulinotropism after prolonged agonist exposure were observed for exendin-4-acyl *versus* exendin-4.
- In direct contrast, acylation affected exendin-phe1’s properties adversely. Exendin-phe1-acyl had reduced signalling potency (and efficacy at the rat GLP-1R) for cAMP accumulation compared to exendin-phe1. This effect could not be compensated for by exendin-phe1-acyl’s preservation of a favourable trafficking profile, as reflected in its reduced insulinotropism *in vitro*. Interestingly, exendin-phe1-acyl’s reduced acute signalling potency would not be predicted by its significantly increased receptor binding affinity compared to exendin-phe1. Although not directly assessed, this suggests that its loss of potency is due to weaker coupling to the $G\alpha_s$ signalling pathway, to the extent that even in β -cells with high levels of GLP-1R expression a full response was not achieved (analogous to the performance of non-acylated exendin-phe1 in neurons).
- Finally, acylation of exendin-asp3 led to increased potency and efficacy in prolonged insulin secretion assays *in vitro*. This is presumably attributable to decreased β -arrestin-2 recruitment and subsequent β -arrestin-2-mediated desensitisation for exendin-asp3-acyl *versus* exendin-asp3, given that the trafficking properties of the two compounds were matched.

Though intriguing, the mechanistic basis for these agonist-specific alterations is unclear and warrants further investigation. A few potential mechanisms may be offered.

- Firstly, the fatty acid chain may directly interact with the GLP-1R to alter agonist orientation and therefore binding and activation parameters. Structural studies modelling receptor-

agonist interactions of the acylated agonists in comparison with unmodified agonists would be informative in this regard.

- Secondly, interactions between the fatty acid and amino acid functional groups in the peptide backbone may stabilise peptide secondary structure and alter activity at the receptor. Peptides cycle through an ensemble of conformations rather than maintaining that required for receptor activation. As such, stabilising α -helices in the peptide secondary structure represents a way of maintaining the biologically relevant conformation and increasing peptide activity [560]. Acylation has been previously reported as a means to achieve this: conjugation of a C16 fatty acid to dual GLP-1R/GCGR agonists stabilised peptide helical secondary structure due to constructive interactions between the fatty acid and peptide functional groups, and consequently increased potency at both receptors [561].
- Finally, the introduction of a lipophilic group may facilitate interactions with the plasma membrane to alter the local concentration of agonist. The notion that the plasma membrane lipid phase may aid peptide-receptor interactions was proposed as early as 1986 (“membrane catalysis” model) [562]; its basis was that a ligand has a much higher probability of interacting with the membrane lipid phase than the embedded receptor fraction (given their relative surface areas). Lipidation may enhance these peptide-membrane interactions, and indeed protein lipidation intracellularly as a post-translational modification is a means for targeting synthesised proteins to the plasma membrane and increasing membrane affinity [563]. A similar phenomenon may occur for extracellularly-located lipidated receptor agonists. Direct evidence for this is sparse, but the following observations lend support.
 - Pepducins are cell-penetrating peptides that act at the GPCR intracellular face or transmembrane domain to alter GPCR activity. Their membrane binding and penetrating ability is attributed to their conjugation to a fatty acid chain [564].
 - Ghrelin exists endogenously in two forms: as active, octanoylated ghrelin, and as the classically considered inactive, non-octanoylated desacyl-ghrelin. However, the latter has been shown to be capable of activating the GHSR *in vitro*, though with potency in the micromolar range compared to ghrelin's nanomolar potency [565]. The biological significance of this supraphysiological GHSR activation by desacyl-ghrelin— if any — is unclear [566]. The acyl group has been directly shown to increase ghrelin's interaction with, and local concentration at the plasma membrane relative to desacyl-ghrelin [547]. It is thought that this raised local concentration facilitates increased interaction with the GHSR, therefore underlying the marked difference in potency between the ghrelin and desacyl-ghrelin, and the existence of biological activity for only the former [547].

Thus, although acylated GLP-1RAs liraglutide and semaglutide are market-leading therapeutics, the effects of acylation upon molecular interactions between the fatty acid group, peptide, GLP-1R and plasma membrane remain poorly characterised. The fact that acylation-induced changes are clearly dependent upon the peptide backbone may make it hard to arrive at a unified theory of its impacts even if identical conjugation conditions are used. However, greater mechanistic understanding would undoubtedly be informative for the design of future acylated GLP-1RAs.

6.3.2 *In vivo* properties of the acylated biased GLP-1RAs in lean mice

As a first point and as anticipated, acylation caused significant half-life protraction *in vivo* relative to the unmodified compounds. This was shown directly for exendin-4 and exendin-4-acyl (Figure 6.14), and indirectly for all compounds by their prolonged physiological effects relative to their unmodified counterparts. Given the stark pharmacokinetic differences between the unmodified and acylated compounds, physiological comparisons were only made between the latter set of agonists for the work in this chapter.

6.3.2.1 Glycaemic effects

Overall, the time-dependent glycaemic effects of the unmodified biased GLP-1RAs were preserved after acylation: acutely, all agonists conferred anti-hyperglycaemic effects, whereas after a delay only exendin-4-acyl and exendin-phe1-acyl provided glycaemic benefits (Figure 6.10), matching the pattern seen with their non-acylated equivalents. However, at equimolar dosing, exendin-phe1-acyl showed weaker acute effects and subsequently failed to outperform exendin-4-acyl in the 72-hour delayed GTT. These changes correlate well with what is seen *in vitro*. Acylation magnified the existing difference in potency between exendin-4 and exendin-phe1 (4.07-fold higher for exendin-4 *versus* exendin-phe1, 18.4-fold higher for exendin-4-acyl *versus* exendin-phe1-acyl; Table 6.1) to an extent that could not be compensated for by exendin-phe1-acyl's reduced β -arrestin recruitment and favourable trafficking profile relative to exendin-4-acyl (Table 5.3). As such, insulinotropic efficacy after prolonged incubation *in vitro* was similar for the two compounds, mirroring their matched performance in delayed GTTs. Nevertheless, it is notable that an agonist with considerably reduced potency and (presumably) acute G_{α_s} signalling efficacy when measured *in vitro* can match the performance of a substantially more potent and efficacious ligand at a reasonable dose *in vivo*. This suggests that the minimal β -arrestin recruitment seen with this ligand is indeed facilitating sustained signalling responses *in vivo* that are not adequately captured using the biochemical measurements in the *in vitro* section. Exendin-4-acyl may also itself benefit from some of the pharmacological attributes previously ascribed to non-acylated exendin-phe1, such as reduced efficacy for β -arrestin recruitment, reduced endocytosis and faster recycling.

By contrast, exendin-asp3-acyl's relative relationship to exendin-4-acyl mimicked that of their unmodified counterparts, with a time-dependent loss of anti-hyperglycaemic efficacy for the former agonist. Again, this corresponded with ligand *in vitro* properties, as exendin-asp3-acyl exhibiting increased β -arrestin recruitment, increased agonist-induced internalisation, reduced GLP-1R recycling, and decreased maximal insulin secretion relative to exendin-4-acyl (Table 5.3). Thus, acylation of the biased GLP-1RAs with a C16 moiety at Lys40 maintains the bias-related glucoregulatory differences between exendin-4-acyl and exendin-asp3-acyl, but seemingly mitigates some of the usual benefits of exendin-phe1, with exendin-phe1-acyl performing similarly to exendin-4-acyl over sustained periods when administered at the same dose. However, this needs to be related to the effects on appetite suppression and the limit this imposes on dosing, as it may be that when given at "equivalently tolerable" doses, a different pattern in glucoregulatory efficacy is observed (analogous to the therapeutic window analysis in Chapter 3).

6.3.2.2 Anorectic effects

Turning to appetite suppression, the relative relationships between the acylated agonists' anorectic effects did not match that of the unmodified compounds. Whereas short- and long-term differences in hypophagia were subtle for non-acylated agonists (Figure 3.3, Figure 3.4), exendin-phe1-acyl and exendin-asp3-acyl were significantly less efficacious than equimolar doses of exendin-4-acyl at all time points (Figure 6.9). Again, this may be expected for exendin-phe1-acyl given that, as discussed directly above, acylation magnifies the difference in potency between exendin-4 and exendin-phe1. However, this does not apply for exendin-asp3-acyl; the relative difference in potency for exendin-4 and exendin-asp3 (1.26-fold higher for the former) is similar to that between exendin-4-acyl and exendin-asp3-acyl (1.59-fold higher for the former). As discussed in section 6.2.6, exendin-asp3-acyl's weaker anorectic efficacy relative to exendin-4-acyl may be due to its greater levels of β -arrestin recruitment (Table 6.1), and sustained concentrations at CNS anorexigenic neurons driving enhanced GLP-1R desensitisation. Admittedly, these desensitisation-related effects are more likely to explain its reduced efficacy at later time points. However, exendin-asp3-acyl also induces significantly less hypophagia than exendin-4-acyl at the 1-hour mark at 2 nmol/kg (perhaps mirroring its subtly reduced anti-hyperglycaemic effect relative to exendin-4-acyl at the same dose in acute GTTs, Figure 6.10). What underlies this observation is unclear. In summary, acylation appears to impact the anorectic effects of both G-protein-biased exendin-phe1-acyl and β -arrestin-biased exendin-asp3-acyl. Whilst this can theoretically be accounted for by the *in vitro* properties of the former, for the latter an explanation likely relies upon a combination of exendin-asp3-acyl's *in vitro* properties (increased β -arrestin-mediated desensitisation) and its enhanced pharmacokinetic parameters.

Though not directly assessed here, equally of interest is a comparison of the relative anorectic effects of the acylated and unmodified compounds. Of note, additional studies from the laboratory suggest that exendin-4-acyl may show marginally weaker anorectic effects relative to exendin-4 at acute time points (data not shown). This is not anticipated from the *in vitro* results, given that: the two compounds have matched potency (Table 6.1); exendin-4-acyl has an "improved" trafficking profile (though the advantages of this would manifest to a greater extent at later time points) (Table 6.5); and exendin-4-acyl is present at higher levels in the plasma at 1 hour (Figure 6.14). However, it could be explained if the "free" drug concentrations differ due to extensive binding to albumin of the acylated form. Beyond this simple explanation, it is possible that exendin-4-acyl exhibits slower access to appetite regulating CNS nuclei; this would not be due to its endocytic profile given that brain uptake is independent of GLP-1R endocytosis (as demonstrated in Chapter 4), but may be due to fatty acid-mediated interactions. Binding to plasma albumin may reduce the free fraction of peptide capable of crossing the BBB/BCSFB, and whilst interactions with the BBB/BCSFB cell plasma membranes may initially assist uptake into these cells, they may subsequently hinder peptide exit and entry into the CSF or brain parenchyma [458]. However, any acute deficit in brain entry is likely compensated for by sustained plasma levels and therefore increased interaction with the BBB/BCSFB over time. Indeed, by 8 hours and for all subsequent time points, exendin-4-acyl is more hypophagic than exendin-4 (data not shown), presumably due to the latter having incurred greater degradation/clearance than the former.

In light of these subtle temporal differences in the anorectic efficacy of exendin-4 and exendin-4-acyl, along with the suggestion that exendin-asp3-acyl's weaker anorectic effects may be related to agonist concentration at CNS appetite-regulating nuclei, it may be informative to assess brain uptake of the

acylated agonists relative to their unmodified forms. This could be achieved by the use of fluorescently-tagged forms of the acylated agonists (provided that dual peptide modification does not overly compromise signalling and trafficking characteristics) in whole brains, or in brain slices. As any difference is likely to be subtle, the increased resolution of the latter approach may be more appropriate.

6.3.3 *In vivo* effects of the acylated biased GLP-1RAs in physiological vs. pathological states

An interesting observation that arose from the work in this chapter was the difference in the relative anti-hyperglycaemic and anorectic effects of the agonists under physiological *versus* pathological conditions (i.e. lean mice and DIO mice, respectively). To reiterate, in studies with DIO mice, doses of the acylated agonists that were “equiactive” for acute (1-hour) appetite suppression were used in attempt to correct for the large differences in potency observed *in vitro* and *in vivo* in lean mice. This marker of “biological potency” was elected as it provides an indication of the degree of nausea (or conversely, tolerability), and is therefore therapeutically relevant given that nausea constitutes the dose-limiting side effect of GLP-1RAs in humans.

Intriguingly, rather than the pathological diet-induced obese state causing common changes to the effects of the agonists (e.g. a global loss of sensitivity to their effects), agonist-specific changes were observed. No impact was observed upon exendin-asp3-acyl's effects, with similar responses obtained for this agonist in both GTTs and food intake studies in lean and DIO mice. By contrast, whilst in lean mice exendin-phe1-acyl had similar anti-hyperglycaemic, and worse anorectic efficacy relative to exendin-4-acyl, in DIO mice it had notably better effects in both physiological tests. Thus, at an equally tolerable dose, exendin-phe1-acyl appears to provide greater glycaemic improvements and anorectic effects to exendin-4-acyl. This is in addition to the significant pharmacokinetic improvement that acylation confers, reducing the required frequency of dosing. Chronic studies are required to assess whether the effects of the agonists, and exendin-phe1-acyl's superior performance persists with repeated administration. These studies are underway, and results indicate that exendin-phe1-acyl does indeed induce significantly greater weight loss than exendin-4-acyl at an equiactive dose, and matched effects at an equimolar dose, despite their large differences in potency (data not shown).

Exendin-phe1-acyl's differential effects under physiological and pathological conditions suggest the occurrence of “dynamic bias”, where the signalling landscape - including membrane composition, the stoichiometric ratio of receptors and signalling components (G-proteins, β -arrestins), and level of spontaneous receptor activity – is modified by physiologic, pathologic or iatrogenic factors, resulting in altered cellular responses to biased agonists [336], [342]. As discussed in section 1.10.1, dynamic bias has been proposed to underlie the failure of angiotensin 1 receptor (AT1R) biased agonist TRV027 in clinical trials for congestive heart failure, with disease-induced signalosome changes negating the clinical relevance of AT1R biased signalling [342]. T2DM and obesity are associated with pathological changes in the pancreas and hypothalamus that may impact GLP-1R biased signalling. At β -cells, chronic hyperglycaemia results in GLP-1R downregulation [548], lipotoxicity disrupts islet connectivity [155], and dyslipidaemia leads to changes in β -cell membrane lipid composition [549]. Likewise, GLP-1R expression is reduced in hypothalami from T2DM patients [554], and exendin-4-mediated hypophagia is attenuated in obese rodents [552], [553].

However, how tissue changes such as these impact signalling responses to the biased GLP-1RAs tested here is unknown. Moreover, why selective changes are observed to exendin-phe1-acyl's biological efficacy is undetermined, but represents an intriguing avenue for investigation. Thus, in addition to the need for careful analysis of the tissue-specific effects of the biased agonists (tissue bias) as discussed in Chapter 5, consideration must also be given to how their signalling is affected in relevant tissues under the obese, diabetic state (dynamic bias).

6.3.4 Summary

This chapter examined the impact of conjugation of a C16 fatty acid to the biased GLP-1RAs. As anticipated, acylation led to significant pharmacokinetic enhancement with corresponding prolongation of anorectic and glycaemic effects. However, acylation impacted the *in vitro* and *in vivo* properties of the agonists differentially, emphasising the influence of existing agonist properties upon subsequent acylation-induced changes. Additionally, the results highlight the impact of pathological state upon the *in vivo* efficacy of the acylated agonists, and thus the importance of assessing responses in a therapeutically-relevant model of T2DM and obesity. Of note, exendin-phe1-acyl may represent a pharmacokinetically improved form of exendin-phe1, with superior anti-hyperglycaemic and anorectic effects to exendin-4-acyl at equally tolerable doses in DIO mice. Further studies are required to confirm this notion.

7 General Discussion

7.1 Introduction

The twin metabolic diseases of T2DM and obesity are global health concerns of growing proportions [2], [567]. There is currently an unmet need for treatments with both high efficacy and a benign side effect profile. The generation of novel therapeutics, or optimisation of existing therapeutics is therefore a priority.

GLP-1RAs are an effective drug class used in the treatment of T2DM, and have recently been licensed as independent anti-obesity medications [235]. GLP-1RAs induce insulin secretion, enhance pancreatic β -cell function, promote weight loss (and therefore insulin sensitivity), and, in cases, improve renal and cardiovascular outcomes [235], [568], [569]. However, they are associated with gastrointestinal side effects, of which nausea is the most common and a major reason for treatment discontinuation [271], [570]. Early stage clinical trials repeatedly evidence high doses of GLP-1RAs to be more effective glycaemically but to be associated with unacceptable side effect profiles [571], [572]. This suggests that the maximum therapeutic potential of GLP-1R agonism has not yet been reached, offering scope for optimisation.

Biased agonism represents a potential means to achieve therapeutic optimisation. It describes the ability of ligands to cause selective activation of particular intracellular pathways by stabilising distinct receptor conformations upon binding [336], [337]. Where cellular responses can be correlated with physiological outcomes, biased agonism can be leveraged to modulate drug effects, biasing ligands towards intracellular pathways associated with desired drug effects (thereby enhancing efficacy), and away from those associated with side effects (thereby improving tolerability). The therapeutic utility of biased agonism has been demonstrated for multiple drug classes, including that of opioid analgesics [348], [349].

It has recently been demonstrated that exendin-4-based GLP-1RAs biased towards G-protein signalling over β -arrestin recruitment exhibit enhanced anti-hyperglycaemic effects [323], [370]. Moreover, G-protein-biased exendin-phe1 displayed matched anorectic and weight-lowering effects to exendin-4 in rodents, but was associated with less nausea [323]. From a therapeutic perspective, G-protein-biased GLP-1RAs may therefore represent ligands with superior anti-hyperglycaemic efficacy, equivalent anorectic efficacy, and greater tolerability in comparison to unbiased GLP-1RAs.

Viewing these data alternatively, exendin-phe1's enhanced effects at pancreatic β -cells did not translate into commensurate increases in anorectic efficacy, suggesting a physiological divergence between the glucoregulatory and anorectic effects of biased GLP-1RAs. This project sought to confirm the existence of this divergence, and investigate its underlying mechanisms.

7.2 Confirmation of the divergent physiological effects of biased GLP-1RAs

The data presented in Chapter 3 substantiate the existence of a divergence between the glycaemic and anorectic effects of exendin-4 and G-protein-biased exendin-phe1, and further extend this to include oppositely, β -arrestin-biased exendin-asp3 (previously only studied *in vitro* [323]). All three

agonists had similar anti-hyperglycaemic effects in acute GTTs but after a 6 hour delay to allow for desensitisation- and trafficking-related differences to emerge, performed differentially, with exendin-phe1 being the most, and exendin-asp3 the least effective (Figure 3.1). These observations were not due to pharmacokinetic differences (Figure 3.2), and are consistent with the respective *in vitro* signalling characteristics of the agonists in MIN6B1 and INS1 823/3 pancreatic β -cell lines [323]. In contrast, the anorectic effects of the biased GLP-1RAs after peripheral administration displayed only subtle time-dependent differences, and were matched overall (Figure 3.3).

Moreover, the physiological divergence was quantitatively established by constructing dose response curves of glycaemic and anorectic responses (Figure 3.4). These experiments confirmed exendin-phe1 to be biased towards anti-hyperglycaemic over anorectic effects relative to exendin-4, with the magnitude of this bias increasing after a delay. Thus, these data provide an *in vivo* correlate of exendin-phe1's *in vitro* G-protein *versus* β -arrestin bias. However, in these experiments exendin-asp3 exhibited similar (Figure 3.4) and not worse (Figure 3.1) delayed glycaemic responses relative to exendin-4, the reasons for which are unclear. Given their matched anorectic effects, exendin-asp3's *in vivo* bias factor was therefore minimal (Figure 3.4). Overall, however, these data strengthen the link between GLP-1RA G-protein bias *in vitro* and bias towards anti-hyperglycaemic effects *in vivo*. Moreover, re-expressing these data as a therapeutic window (Figure 3.4 H) revealed exendin-phe1 to have much wider separation between doses inducing beneficial anti-hyperglycaemic effects, and those causing acute appetite suppression, a proxy of nausea. They therefore support previous findings of exendin-phe1 being a more tolerable GLP-1RA relative to exendin-4 [323]. This conclusion comes with the caveat that acute appetite suppression may not be the optimal measure of nausea. However, examining other behavioural rodent correlates of nausea (e.g. pica, condition taste aversion, behavioural satiety sequence) with the wide range of doses assessed here would have been impractical. It may be interesting to conduct said behavioural tests for selected doses to assess whether, in contrast to G-protein-biased exendin-phe1, β -arrestin-biased exendin-asp3 induces more nausea than exendin-4. Studies such as these may reveal differences in the qualitative nature of the anorectic effects of the biased agonists. This is of heightened interest given the suggestion in Chapter 4 that exendin-phe1 may reach CNS regions associated with GLP-1R-induced hypophagia without nausea to a greater extent than the other agonists (see Section 4.4.3).

Two potential explanations were offered for the observed physiological divergence:

1. Differences in *access* to CNS-located appetite regulatory centres due to the differential endocytic profiles of the biased GLP-1RAs; or
2. The existence of *tissue-specific signalling* between the brain and pancreas.

In the course of investigation, a third potential hypothesis was formulated:

3. Differences in the overall *tissue access* of the biased GLP-1RAs between the brain and pancreas.

In short, the data in this thesis suggest that tissue-specific signalling underlies the physiological divergence between the glycaemic and anorectic effects of the biased GLP-1RAs. The key findings that led to this conclusion are reiterated below.

7.3 Brain and pancreatic access of GLP-1R ligands

Experiments examining the brain and pancreatic uptake of fluorescently-tagged forms of the biased GLP-1RAs evidenced the following three key findings:

1. Access to CNS neurons was similar for each agonist.
2. Ligand spread beyond CVOs and into BBB-protected locations (the ARC, NTS, and CSF) was not reduced with ligands showing decreased GLP-1R endocytosis.
3. Overall GLP-1R ligand access to CNS sites was similar to the pancreas.

Findings 1 and 2 argue against the hypothesis that the biased GLP-1RAs exhibit differential access to central appetite-regulating nuclei. The basis for this hypothesis was that brain uptake of GLP-1RAs might be mediated by GLP-1R endocytosis, and therefore endocytosis-deficient exendin-phe1 would show reduced uptake relative to endocytosis-prone exendin-4 and exendin-asp3. This possibility has been recently proposed for GLP-1R following confirmation of a similar phenomenon for leptin [179], [391]. If applicable to GLP-1R ligands, it would explain the absence of exendin-phe1's time-dependent signalling benefit in the context of appetite suppression. However, the presented data do not support this reasoning, and instead evidence agonist access to both CVOs and BBB-protected regions to be similar, and unrelated to endocytic properties (Figure 4.13, Figure 4.14, Figure 4.15). Moreover, the uptake of non-internalising antagonist exendin(9-39) into both BBB-protected brain regions (Figure 4.18, Figure 4.19, Figure 4.20) and the CSF (Figure 4.17) was equivalent to exendin-4, and therefore strongly suggests that brain uptake of GLP-1R ligands does not occur by receptor-mediated endocytosis. This aligns with previous findings that the brain entry of a degradation-resistant form of GLP-1 is not affected by the co-administration of exendin(9-39), that exendin-4 crosses the BBB with rapid and non-saturable kinetics, and that exendin-4 displays physicochemical properties permissive for diffusion across the BBB [194], [423]. Thus, evidence to date suggests that exendin-4-based GLP-1RAs enter the brain passively via transcellular diffusion (see Section 4.4.5 for more detail). However, as extensively discussed in Section 4.4.5, *in vitro* validation of this process in cells of the BBB/BCSFB for both exendin-4-based GLP-1RAs and other GLP-1RAs (e.g. liraglutide) would be invaluable, particularly as the mechanistic basis for GLP-1RA brain uptake is poorly understood [453].

With regard to Finding 3, the possibility of differences in tissue access between the brain and the pancreas was raised by two observations. Firstly, the large difference in agonist dose required to elicit glycaemic and anorectic effects (Figure 3.4, Table 3.1), and secondly, the observation that levels of GLP-1R ligands detected in the CSF were approximately 4% of plasma concentration (Figure 4.17). One interpretation of these data is that the level of agonist that reaches CNS neurons is significantly lower than that at pancreatic islets. As agonist-related homologous desensitisation tends to be most apparent at high doses, it may be the case that only within the pancreas are bias-related differences in GLP-1R desensitisation able to be manifested. Again, if true, this would potentially explain the agonists' broadly equivalent anorectic efficacy, especially at later time points when desensitisation-related differences are observed in glycaemic responses. However, the average fluorescence intensity in exendin-4-VT750-positive brain regions and pancreatic islets was similar (Figure 4.18; Finding 3), thereby excluding potential tissue differences in agonist bioavailability.

Assessing agonist concentration in the brain and pancreas via another means in order to corroborate this conclusion would be worthwhile, particularly so given the requirement of a high dose of VT750-

tagged agonist for adequate detection (as discussed in Section 4.4.7). For example, agonist concentrations in the brain could be monitored *in vivo* over time by microdialysis of the interstitial fluid in regions of interest (e.g. the ARC), as has been previously described for leptin in the hypothalamus [573], [574]. Pancreatic microdialysis is less well established, though has been described in rats [575]. Alternatively, peptide concentration could be assayed in tissue homogenates after peripheral administration [439], [576]. Either of these approaches would potentially facilitate the measurement of agonist below the level of detection by fluorescence in whole brains (as discussed in Section 4.4.4) and may also allow for the testing of lower and more physiologically relevant peripheral doses of agonist (depending upon the sensitivity of the assay used for peptide detection). Conversely, these approaches inherently lack spatial resolution, which is not ideal given the fact that ligand is likely to be highly concentrated in specific locations within the sampled organ (GLP-1R-expressing pancreatic β -cells and GLP-1R-expressing neurons). It may be the case that different and complementary information is provided by both approaches, with the current imaging strategy providing insights into the distribution of receptor-bound ligand, with more relevance to receptor pharmacology and observed biological effects, and the alternative approaches indicating the overall tissue concentration, irrespective of whether it relates to the cells that express GLP-1R.

7.4 Tissue-specific signalling of biased GLP-1RAs

The exclusion of hypotheses based upon differential agonist access to CNS appetite regulating centres, or general differences in access for all agonists to the brain *versus* pancreas, lent substantial support to tissue-specific signalling as a novel explanation for the physiological divergence between the biased GLP-1RAs. Subsequent investigations comparing the signalling responses to the biased GLP-1RAs in *in vitro* and *ex vivo* ARC neurons to those in MIN6B1 pancreatic β -cells led to the following key findings:

1. Receptor expression was significantly lower in neuronal *versus* pancreatic β -cell lines (Figure 5.2).
2. GLP-1RA potency for acute responses was notably lower in neuronal *versus* pancreatic β -cell lines (Table 5.1).
3. Exendin-phe1 showed significantly reduced signalling efficacy in neurons *versus* pancreatic β -cells at both short- and long-term time points (Figure 5.1, Figure 5.2).

Importantly, these *in vitro* findings appear to marry well with *in vivo* observations. Finding 2 correlates with the fact that considerably higher doses of agonist are required to elicit anorectic *versus* glycaemic responses *in vivo* (Figure 3.4, Table 3.1). Thus the differential “biological potency” of the agonists appears to be related to intrinsic cellular properties as opposed to differences in agonist concentration experienced by *in situ* pancreatic β -cells and anorexigenic neurons. Finding 2 may, at least in part, be explained by Finding 1. In addition, GLP-1R coupling to downstream signalling effectors, or signal amplification in neurons may be weaker than that in pancreatic β -cells. Admittedly, differences in agonist potency *in vitro* were observed using immortalised cell lines that may artificially accentuate differences in GLP-1R expression. However, supporting the relevancy of findings in cell lines, large differences in GLP-1R expression level were also observed between primary vagal neurons and sorted pancreatic β -cells [141], and also between pancreatic islet tissue *versus* the hypothalamus and brainstem [492].

Investigation here has focussed upon CNS neurons, and in particular neurons of the ARC given strong evidence for their importance in mediating the anorectic effects of GLP-1RAs [179], [465], [466]. As noted in Section 5.4.4, examination of GLP-1R expression levels and signalling responses could be extended to alternative GLP-1R expressing regions in the CNS (e.g. the DVC) through the use of transgenic mouse lines with co-expression of the GLP-1R with fluorescent reporters. Moreover, some studies suggest that vagal GLP-1Rs contribute to the anorectic effects of pharmacological GLP-1RAs (though to a lesser extent than central GLP-1Rs) [163], [196]. It may therefore be pertinent to see whether findings in CNS neurons extend to those of the peripheral nervous system. The low receptor expression in primary vagal neurons [141] noted above suggests that this may be the case.

Turning to Finding 3, the data presented in this thesis evidence differences in the magnitude of response to the biased GLP-1RAs in pancreatic β -cells *versus* neurons. Specifically, exendin-phe1 exhibited significantly reduced signalling efficacy relative to the other agonists both acutely (as measured by cAMP accumulation; Figure 5.1) as well as at later time points (as assessed by RNAseq; Figure 5.2). This agonist-specific neuronal difference appears to be related to exendin-phe1's nature as a partial agonist for $G\alpha_s$ recruitment, meaning that under conditions of low GLP-1R expression in neurons it fails to engender a maximal intracellular cAMP response. By contrast, in pancreatic β -cells where GLP-1R expression and signal amplification in the $G\alpha_s$ pathway is high (Figure 5.2), exendin-phe1 is able to act as a full agonist. Over time in β -cells, exendin-phe1's reduced propensity for desensitisation and favourable trafficking profile manifest as enhanced efficacy relative to the other agonists at later time points; in neurons however, these beneficial properties fail to compensate for exendin-phe1's marked loss of efficacy. Again, these *in vitro* observations align with *in vivo* results, as exendin-phe1 manifests time-dependent, superior anti-hyperglycaemic, but not anorectic responses. Thus, differences in the signalling efficacy of the biased GLP-1RAs in anorexigenic neurons and pancreatic β -cells appears to underlie, at least in part, their divergent anorectic and glycaemic effects.

As detailed in Section 5.4.4, further investigation is warranted to confirm the existence of differential tissue responses to the biased GLP-1RAs and to probe its mechanistic basis. To reiterate, some important experiments would include: recapitulating differential responses in primary neuronal and pancreatic β -cells in parallel assays, assessing the level of GLP-1R expression in isolated fractions of GLP-1R-expressing primary cells, identifying a single readout for both short- and long-term responses in neurons (e.g. neuropeptide release) to facilitate more rigorous characterisation of temporal differences in the signalling of the biased GLP-1RAs, and testing the relationship between GLP-1R expression level and response to the biased GLP-1RAs.

In summary, the presented data evidence the influence of cellular context upon responses to the biased GLP-1RAs, and support the existence of tissue bias between the brain and pancreas. In particular, the data suggests that a major difference in the signalling landscape of neurons *versus* β -cells is the level of GLP-1R expression, and that this may, at least in part, underlie the differences in the agonist dose required for glycaemic and anorectic effects *in vivo*, and the differential efficacy of G-protein-biased GLP-1RAs for the two physiological responses. Such tissue bias has therapeutic relevance given that both beneficial effects (insulintropism and weight loss) and side effects (e.g. nausea, vomiting) of GLP-1RAs are mediated by β -cells and neurons. Understanding and optimising responses at the two tissues in a "multi-systems" approach should therefore aid the therapeutic translation of biased GLP-1RAs. As a final point, pharmacological GLP-1RAs have impacts upon

other tissues, including the heart and kidneys [568], [569]. Investigation could thus be extended by assessing GLP-1R expression levels and signalling responses to the biased GLP-1RAs in cardiac, renal, and other tissues, in order to generate a more holistic picture of tissue-specific GLP-1R biased agonism.

7.5 Optimisation of biased GLP-1R agonism in the pathological state

In addition to demonstrating the existence of tissue-specific responses, the data presented in this thesis suggest that signalling responses to biased GLP-1RAs may differ between physiological and pathological states. In Chapter 6, acylated forms of the biased GLP-1RAs with significantly improved pharmacokinetics (Figure 6.14) were characterised *in vitro* (Table 6.5), and *in vivo* in both lean mice (Figure 6.9, Figure 6.10) as well as in a mouse model of T2DM and obesity (Figure 6.11, Figure 6.12). Of particular interest is the difference in exendin-phe1-acyl's properties in these three systems. *In vitro*, exendin-phe1-acyl exhibited a significant loss of potency relative to its non-acylated form (Table 6.1), thereby magnifying the existing difference in signalling potency relative to exendin-4-acyl (which itself exhibited comparable potency to non-acylated exendin-4). Exendin-phe1-acyl's decreased potency translated, in lean mice, into weaker overall anorectic effects, weaker acute anti-hyperglycaemic effects, and equivalent delayed anti-hyperglycaemic effects relative to exendin-4-acyl (Figure 6.9, Figure 6.10). Whilst still supporting the general concept that GLP-1RAs with reduced β -arrestin recruitment tend to exhibit time-dependent improvements in their anti-hyperglycaemic efficacy, the therapeutic impact was less evident than with non-acylated exendin-phe1 and exendin-4, presumably due to the larger impact of acylation on potency with exendin-phe1. However, in DIO mice, exendin-phe1-acyl had significantly better anorectic and anti-hyperglycaemic effects at delayed time points relative to exendin-4-acyl at an equally tolerable dose (Figure 6.11, Figure 6.12). Moreover, ongoing work in the laboratory has demonstrated that differences in anorectic efficacy between the agonists appear to persist with chronic administration in DIO mice, leading to superior weight-lowering effects for exendin-phe1-acyl (data not shown).

This “restoration” of the anticipated time-dependent advantage of exendin-phe1-acyl in DIO mice reveals that the pathological diabetic, obese state appears to alter the biological efficacy of biased GLP-1RAs differentially, rather than leading to equivalent changes in their effects. Thus, these data suggest the existence of “dynamic bias” [342] in GLP-1R agonism between physiological and pathological states. As discussed in Section 6.3.3, the exact nature of the pathological changes in pancreatic β -cells and anorexigenic neurons and how they lead to agonist-specific changes is yet unclear, though represents an interesting and therapeutically-relevant avenue for investigation. Future work could therefore involve characterising responses to both acylated and unmodified forms of the agonists in *in vitro* or *ex vivo* β -cell and neuronal models of T2DM/obesity. This could be achieved by exposing cells or normal tissue to sustained glucoxic or lipotoxic insults *in vitro*, or, as a more representative pathophysiological model, by harvesting primary cells and tissue from mouse models of T2DM/obesity. Given that exendin-phe1-acyl's *in vitro* and *in vivo* properties under physiological conditions were not predictive of its superior effects in DIO mice, it may be pertinent in future work to assess the signalling responses to biased GLP-1RAs in *in vitro* models of T2DM/obesity prior to their testing in *in vivo* models.

7.6 Summary

In summary, the work of this thesis confirms the existence of a physiological divergence between the glycaemic and anorectic effects of exendin-4, exendin-phe1, and exendin-asp3. It suggests that differential access to central appetite-regulating centres, or between the brain and pancreas, does not account for this divergence, and, additionally, evidences the brain uptake of exendin-4-based ligands to be independent of GLP-1R endocytosis at the BBB/BCSFB. Instead of differences in agonist access, it points towards tissue-specific signalling responses between the pancreas and brain being the underlying cause of the observed divergence. More broadly, it highlights the importance of assessing responses to biased GLP-1RAs in the multiple tissue types relevant to their therapeutic and side effects, and in models of T2DM and obesity given indication of signalling changes specific to the pathological state.

8 Appendix 1

Unmodified Compounds

	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
Ex4	H	G	E	G	T	F	T	S	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	P	S	NH2
Ex-phe1	F	G	E	G	T	F	T	S	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	P	S	NH2
Ex-asp3	H	G	D	G	T	F	T	S	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	P	S	NH2
Ex(9-39)									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	P	S	NH2

Pra12-modified Compounds

	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
Ex4-pra12	H	G	E	G	T	F	T	S	D	L	S	Pra	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	P	S	NH2
Ex-phe1-pra12	F	G	E	G	T	F	T	S	D	L	S	Pra	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	P	S	NH2
Ex-asp3-pra12	H	G	D	G	T	F	T	S	D	L	S	Pra	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	P	S	NH2
Ex(9-39)-pra12									D	L	S	Pra	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	P	S	NH2

sCy7-tagged Compounds

	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
Ex4-pra12-sCy7	H	G	E	G	T	F	T	S	D	L	S	Pra-sCy7	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	P	S	NH2
Ex-phe1-pra12-sCy7	F	G	E	G	T	F	T	S	D	L	S	Pra-sCy7	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	P	S	NH2
Ex-asp3-pra12-sCy7	H	G	D	G	T	F	T	S	D	L	S	Pra-sCy7	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	P	S	NH2
Ex(9-39)-pra12-sCy7									D	L	S	Pra-sCy7	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	P	S	NH2

Vt750-tagged Compounds

	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	
Ex4-pra12-VT750	H	G	E	G	T	F	T	S	D	L	S	Pra-VT750	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	P	S	NH2	
Ex-phe1-pra12-VT750	F	G	E	G	T	F	T	S	D	L	S	Pra-VT750	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	P	S	NH2	
Ex-asp3-pra12-VT750	H	G	D	G	T	F	T	S	D	L	S	Pra-VT750	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	P	S	NH2	
Ex(9-39)-pra12-VT750										D	L	S	Pra-VT750	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	P	S	NH2

LUXendins

	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
LUXendin645									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	P	C-Cy5	NH2

Acylated Compounds

	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
Ex4-acyl	H	G	E	G	T	F	T	S	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	P	S	K-C16	NH2
Ex-phe1-acyl	F	G	E	G	T	F	T	S	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	P	S	K-C16	NH2
Ex-asp3-acyl	H	G	D	G	T	F	T	S	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	P	S	K-C16	NH2

9 References

- [1] R. A. DeFronzo *et al.*, "Type 2 diabetes mellitus," *Nat. Rev. Dis. Prim.*, vol. 1, no. 1, p. 15019, Dec. 2015.
- [2] NCD Risk Factor Collaboration (NCD-RisC), "Worldwide trends in diabetes since 1980: a pooled analysis of 751 population-based studies with 4.4 million participants.,", *Lancet (London, England)*, vol. 387, no. 10027, pp. 1513–30, Apr. 2016.
- [3] N. Hex, C. Bartlett, D. Wright, M. Taylor, and D. Varley, "Estimating the current and future costs of Type 1 and Type 2 diabetes in the UK, including direct health costs and indirect societal and productivity costs.,", *Diabet. Med.*, vol. 29, no. 7, pp. 855–62, Jul. 2012.
- [4] A. Abdullah, A. Peeters, M. de Courten, and J. Stoelwinder, "The magnitude of association between overweight and obesity and the risk of diabetes: a meta-analysis of prospective cohort studies.,", *Diabetes Res. Clin. Pract.*, vol. 89, no. 3, pp. 309–19, Sep. 2010.
- [5] E. W. Gregg *et al.*, "Association of an intensive lifestyle intervention with remission of type 2 diabetes.,", *JAMA*, vol. 308, no. 23, pp. 2489–96, Dec. 2012.
- [6] E. S. Schellenberg, D. M. Dryden, B. Vandermeer, C. Ha, and C. Korownyk, "Lifestyle interventions for patients with and at risk for type 2 diabetes: a systematic review and meta-analysis.,", *Ann. Intern. Med.*, vol. 159, no. 8, pp. 543–51, Oct. 2013.
- [7] E. Burgess, P. Hassmén, and K. L. Pumpa, "Determinants of adherence to lifestyle intervention in adults with obesity: a systematic review.,", *Clin. Obes.*, vol. 7, no. 3, pp. 123–135, Jun. 2017.
- [8] J. Thrasher, "Pharmacologic Management of Type 2 Diabetes Mellitus: Available Therapies," *Am. J. Cardiol.*, vol. 120, no. 1, pp. S4–S16, Jul. 2017.
- [9] R. Padwal, S. K. Li, and D. C. W. Lau, "Long-term pharmacotherapy for obesity and overweight.,", *Cochrane database Syst. Rev.*, no. 3, p. CD004094, 2004.
- [10] L. Sjöström *et al.*, "Association of bariatric surgery with long-term remission of type 2 diabetes and with microvascular and macrovascular complications.,", *JAMA*, vol. 311, no. 22, pp. 2297–304, Jun. 2014.
- [11] L. Sjöström *et al.*, "Effects of bariatric surgery on mortality in Swedish obese subjects.,", *N. Engl. J. Med.*, vol. 357, no. 8, pp. 741–52, Aug. 2007.
- [12] E. Rausa, L. Bonavina, E. Asti, M. Gaeta, and C. Ricci, "Rate of Death and Complications in Laparoscopic and Open Roux-en-Y Gastric Bypass. A Meta-analysis and Meta-regression Analysis on 69,494 Patients.,", *Obes. Surg.*, vol. 26, no. 8, pp. 1956–63, 2016.
- [13] K. Dogan, J. Homan, E. O. Aarts, H. de Boer, C. J. H. M. van Laarhoven, and F. J. Berends, "Long-term nutritional status in patients following Roux-en-Y gastric bypass surgery," *Clin. Nutr.*, vol. 37, no. 2, pp. 612–617, Apr. 2018.
- [14] V. Raverdy *et al.*, "Incidence and Predictive Factors of Postprandial Hyperinsulinemic Hypoglycemia After Roux-en-Y Gastric Bypass: A Five year Longitudinal Study.,", *Ann. Surg.*, vol. 264, no. 5, pp. 878–885, Nov. 2016.
- [15] C. J. Lee, T. T. Brown, M. Schweitzer, T. Magnuson, and J. M. Clark, "The incidence and risk factors associated with developing symptoms of hypoglycemia after bariatric surgery.,", *Surg. Obes. Relat. Dis.*, vol. 14, no. 6, pp. 797–802, 2018.
- [16] . National Institute of Health and Care, "Obesity: identification, assessment and management," 2014.
- [17] C. W. le Roux *et al.*, "Gut hormone profiles following bariatric surgery favor an anorectic state, facilitate weight loss, and improve metabolic parameters.,", *Ann. Surg.*, vol. 243, no. 1, pp. 108–14, Jan. 2006.
- [18] S. L. Aronoff, K. Berkowitz, B. Shreiner, and L. Want, "Glucose Metabolism and Regulation: Beyond Insulin and Glucagon," *Diabetes Spectr.*, vol. 17, no. 3, pp. 183–190, Jul. 2004.
- [19] P. Newsholme, V. Cruzat, F. Arfuso, and K. Keane, "Nutrient regulation of insulin secretion and action," *J. Endocrinol.*, vol. 221, no. 3, pp. R105–R120, Jun. 2014.
- [20] J. J. Holst, N. J. Wewer Albrechtsen, J. Pedersen, and F. K. Knop, "Glucagon and Amino Acids Are Linked in a Mutual Feedback Cycle: The Liver- α -Cell Axis," *Diabetes*, vol. 66, no. 2, pp. 235–240, Feb. 2017.
- [21] T. D. Müller, B. Finan, C. Clemmensen, R. D. DiMarchi, and M. H. Tschöp, "The New Biology and Pharmacology of Glucagon," *Physiol. Rev.*, vol. 97, no. 2, pp. 721–766, Apr. 2017.
- [22] G. A. Rutter, "Regulating Glucagon Secretion: Somatostatin in the Spotlight," *Diabetes*, vol. 58, no. 2, pp. 299–301, Feb. 2009.
- [23] B. Moore, E. S. Edie, and J. H. Abram, "On the treatment of Diabetus mellitus by acid extract

- of Duodenal Mucous Membrane," *Biochem. J.*, vol. 1, no. 1, pp. 28–38, Jan. 1906.
- [24] H. ELRICK, L. STIMMLER, C. J. HLAD, and Y. ARAI, "Plasma Insulin Response to Oral and Intravenous Glucose Administration," *J. Clin. Endocrinol. Metab.*, vol. 24, no. 10, pp. 1076–1082, Oct. 1964.
- [25] M. A. NAUCK *et al.*, "Incretin Effects of Increasing Glucose Loads in Man Calculated from Venous Insulin and C-Peptide Responses*," *J. Clin. Endocrinol. Metab.*, vol. 63, no. 2, pp. 492–498, Aug. 1986.
- [26] J. J. Holst, "The incretin system in healthy humans: The role of GIP and GLP-1," *Metabolism*, vol. 96, pp. 46–55, Jul. 2019.
- [27] Y. Seino, M. Fukushima, and D. Yabe, "GIP and GLP-1, the two incretin hormones: Similarities and differences," *J. Diabetes Investig.*, vol. 1, no. 1–2, pp. 8–23, Feb. 2010.
- [28] K. Moens *et al.*, "Expression and Functional Activity of Glucagon, Glucagon-Like Peptide I, and Glucose-Dependent Insulinotropic Peptide Receptors in Rat Pancreatic Islet Cells," *Diabetes*, vol. 45, no. 2, pp. 257–261, Feb. 1996.
- [29] B. Kreymann, M. A. Ghatei, G. Williams, and S. R. Bloom, "GLUCAGON-LIKE PEPTIDE-1 7-36: A PHYSIOLOGICAL INCRETIN IN MAN," *Lancet*, vol. 330, no. 8571, pp. 1300–1304, Dec. 1987.
- [30] M. A. Nauck, E. Bartels, C. Orskov, R. Ebert, and W. Creutzfeldt, "Additive insulinotropic effects of exogenous synthetic human gastric inhibitory polypeptide and glucagon-like peptide-1-(7-36) amide infused at near-physiological insulinotropic hormone and glucose concentrations," *J. Clin. Endocrinol. Metab.*, vol. 76, no. 4, pp. 912–917, Apr. 1993.
- [31] J. P. Raufman, L. Singh, G. Singh, and J. Eng, "Truncated glucagon-like peptide-1 interacts with exendin receptors on dispersed acini from guinea pig pancreas. Identification of a mammalian analogue of the reptilian peptide exendin-4," *J. Biol. Chem.*, vol. 267, no. 30, pp. 21432–7, Oct. 1992.
- [32] C. M. Edwards *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*, vol. 48, no. 1, pp. 86–93, Jan. 1999.
- [33] L. S. Gasbjerg *et al.*, "GIP(3-30)NH₂ is an efficacious GIP receptor antagonist in humans: a randomised, double-blinded, placebo-controlled, crossover study," *Diabetologia*, vol. 61, no. 2, pp. 413–423, Feb. 2018.
- [34] L. S. Gasbjerg *et al.*, "Separate and Combined Glucometabolic Effects of Endogenous Glucose-Dependent Insulinotropic Polypeptide and Glucagon-like Peptide 1 in Healthy Individuals," *Diabetes*, vol. 68, no. 5, pp. 906–917, May 2019.
- [35] M. A. Nauck, M. M. Heimesaat, C. Orskov, J. J. Holst, R. Ebert, and W. Creutzfeldt, "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.*, vol. 91, no. 1, pp. 301–307, Jan. 1993.
- [36] T. Vilsbøll *et al.*, "The Pathophysiology of Diabetes Involves a Defective Amplification of the Late-Phase Insulin Response to Glucose by Glucose-Dependent Insulinotropic Polypeptide—Regardless of Etiology and Phenotype," *J. Clin. Endocrinol. Metab.*, vol. 88, no. 10, pp. 4897–4903, Oct. 2003.
- [37] "2. Classification and Diagnosis of Diabetes," *Diabetes Care*, vol. 38, no. Supplement_1, pp. S8–S16, Jan. 2015.
- [38] G. Willemsen *et al.*, "The Concordance and Heritability of Type 2 Diabetes in 34,166 Twin Pairs From International Twin Registers: The Discordant Twin (DISCOTWIN) Consortium," *Twin Res. Hum. Genet.*, vol. 18, no. 6, pp. 762–771, Dec. 2015.
- [39] A. P. Morris *et al.*, "Large-scale association analysis provides insights into the genetic architecture and pathophysiology of type 2 diabetes," *Nat. Genet.*, vol. 44, no. 9, pp. 981–90, Sep. 2012.
- [40] C. Fuchsberger *et al.*, "The genetic architecture of type 2 diabetes," *Nature*, vol. 536, no. 7614, pp. 41–47, Aug. 2016.
- [41] G. da Silva Xavier *et al.*, "TCF7L2 Regulates Late Events in Insulin Secretion From Pancreatic Islet -Cells," *Diabetes*, vol. 58, no. 4, pp. 894–905, Apr. 2009.
- [42] J. Flannick *et al.*, "Loss-of-function mutations in SLC30A8 protect against type 2 diabetes," *Nat. Genet.*, vol. 46, no. 4, pp. 357–363, Apr. 2014.
- [43] R. H. Unger and S. Grundy, "Hyperglycaemia as an inducer as well as a consequence of impaired islet cell function and insulin resistance: implications for the management of diabetes," *Diabetologia*, vol. 28, no. 3, pp. 119–21, Mar. 1985.
- [44] Y. Lee, H. Hirose, M. Ohneda, J. H. Johnson, J. D. McGarry, and R. H. Unger, "Beta-cell

- lipotoxicity in the pathogenesis of non-insulin-dependent diabetes mellitus of obese rats: impairment in adipocyte-beta-cell relationships.," *Proc. Natl. Acad. Sci.*, vol. 91, no. 23, pp. 10878–10882, Nov. 1994.
- [45] S. Kashyap *et al.*, "A Sustained Increase in Plasma Free Fatty Acids Impairs Insulin Secretion in Nondiabetic Subjects Genetically Predisposed to Develop Type 2 Diabetes," *Diabetes*, vol. 52, no. 10, pp. 2461–2474, Oct. 2003.
 - [46] Y. Ohno, N. Aoki, and A. Nishimura, "In vitro production of interleukin-1, interleukin-6, and tumor necrosis factor-alpha in insulin-dependent diabetes mellitus.," *J. Clin. Endocrinol. Metab.*, vol. 77, no. 4, pp. 1072–1077, Oct. 1993.
 - [47] D. R. Laybutt *et al.*, "Endoplasmic reticulum stress contributes to beta cell apoptosis in type 2 diabetes," *Diabetologia*, vol. 50, no. 4, pp. 752–763, Mar. 2007.
 - [48] M. Bensellam, D. R. Laybutt, and J.-C. Jonas, "The molecular mechanisms of pancreatic β -cell glucotoxicity: Recent findings and future research directions," *Mol. Cell. Endocrinol.*, vol. 364, no. 1–2, pp. 1–27, Nov. 2012.
 - [49] R. A. Ritzel, J. J. Meier, C.-Y. Lin, J. D. Veldhuis, and P. C. Butler, "Human Islet Amyloid Polypeptide Oligomers Disrupt Cell Coupling, Induce Apoptosis, and Impair Insulin Secretion in Isolated Human Islets," *Diabetes*, vol. 56, no. 1, pp. 65–71, Jan. 2007.
 - [50] R. A. DeFronzo, E. Ferrannini, and D. C. Simonson, "Fasting hyperglycemia in non-insulin-dependent diabetes mellitus: contributions of excessive hepatic glucose production and impaired tissue glucose uptake.," *Metabolism*, vol. 38, no. 4, pp. 387–95, Apr. 1989.
 - [51] L. C. Groop *et al.*, "Glucose and free fatty acid metabolism in non-insulin-dependent diabetes mellitus. Evidence for multiple sites of insulin resistance.," *J. Clin. Invest.*, vol. 84, no. 1, pp. 205–213, Jul. 1989.
 - [52] E. Ferrannini *et al.*, "The disposal of an oral glucose load in patients with non-insulin-dependent diabetes.," *Metabolism*, vol. 37, no. 1, pp. 79–85, Jan. 1988.
 - [53] I. Magnusson, D. L. Rothman, L. D. Katz, R. G. Shulman, and G. I. Shulman, "Increased rate of gluconeogenesis in type II diabetes mellitus. A ^{13}C nuclear magnetic resonance study.," *J. Clin. Invest.*, vol. 90, no. 4, pp. 1323–1327, Oct. 1992.
 - [54] M. Matsuda *et al.*, "Glucagon dose-response curve for hepatic glucose production and glucose disposal in type 2 diabetic patients and normal individuals.," *Metabolism*, vol. 51, no. 9, pp. 1111–9, Sep. 2002.
 - [55] A. D. Baron, L. Schaeffer, P. Shragg, and O. G. Kolterman, "Role of Hyperglucagonemia in Maintenance of Increased Rates of Hepatic Glucose Output in Type II Diabetics," *Diabetes*, vol. 36, no. 3, pp. 274–283, Mar. 1987.
 - [56] G. I. Shulman, D. L. Rothman, T. Jue, P. Stein, R. A. DeFronzo, and R. G. Shulman, "Quantitation of Muscle Glycogen Synthesis in Normal Subjects and Subjects with Non-Insulin-Dependent Diabetes by ^{13}C Nuclear Magnetic Resonance Spectroscopy," *N. Engl. J. Med.*, vol. 322, no. 4, pp. 223–228, Jan. 1990.
 - [57] J. E. Gerich, "Role of the kidney in normal glucose homeostasis and in the hyperglycaemia of diabetes mellitus: therapeutic implications," *Diabet. Med.*, vol. 27, no. 2, pp. 136–142, Feb. 2010.
 - [58] Diabetes Prevention Program Research Group, "Long-term effects of lifestyle intervention or metformin on diabetes development and microvascular complications over 15-year follow-up: the Diabetes Prevention Program Outcomes Study.," *lancet. Diabetes Endocrinol.*, vol. 3, no. 11, pp. 866–75, Nov. 2015.
 - [59] K. I. Galaviz, M. B. Weber, A. Straus, J. S. Haw, K. M. V. Narayan, and M. K. Ali, "Global Diabetes Prevention Interventions: A Systematic Review and Network Meta-analysis of the Real-World Impact on Incidence, Weight, and Glucose," *Diabetes Care*, vol. 41, no. 7, pp. 1526–1534, Jul. 2018.
 - [60] K.-S. Kim, R. J. Seeley, and D. A. Sandoval, "Signalling from the periphery to the brain that regulates energy homeostasis," *Nat. Rev. Neurosci.*, vol. 19, no. 4, pp. 185–196, Apr. 2018.
 - [61] K. Timper and J. C. Brüning, "Hypothalamic circuits regulating appetite and energy homeostasis: pathways to obesity," *Dis. Model. Mech.*, vol. 10, no. 6, pp. 679–689, Jun. 2017.
 - [62] T. M. Hahn, J. F. Breininger, D. G. Baskin, and M. W. Schwartz, "Coexpression of AgRP and NPY in fasting-activated hypothalamic neurons," *Nat. Neurosci.*, vol. 1, no. 4, pp. 271–272, Aug. 1998.
 - [63] E. Gropp *et al.*, "Agouti-related peptide-expressing neurons are mandatory for feeding," *Nat. Neurosci.*, vol. 8, no. 10, pp. 1289–1291, Oct. 2005.
 - [64] K. G. Mountjoy, M. T. Mortrud, M. J. Low, R. B. Simerly, and R. D. Cone, "Localization of the melanocortin-4 receptor (MC4-R) in neuroendocrine and autonomic control circuits in the

- brain.," *Mol. Endocrinol.*, vol. 8, no. 10, pp. 1298–1308, Oct. 1994.
- [65] W. Fan, B. A. Boston, R. A. Kesterson, V. J. Hruby, and R. D. Cone, "Role of melanocortineric neurons in feeding and the agouti obesity syndrome," *Nature*, vol. 385, no. 6612, pp. 165–168, Jan. 1997.
 - [66] J. N. Betley, Z. F. H. Cao, K. D. Ritola, and S. M. Sternson, "Parallel, Redundant Circuit Organization for Homeostatic Control of Feeding Behavior," *Cell*, vol. 155, no. 6, pp. 1337–1350, Dec. 2013.
 - [67] M. L. Wolak, M. R. DeJoseph, A. D. Cator, A. S. Mokashi, M. S. Brownfield, and J. H. Urban, "Comparative distribution of neuropeptide Y Y1 and Y5 receptors in the rat brain by using immunohistochemistry," *J. Comp. Neurol.*, vol. 464, no. 3, pp. 285–311, Sep. 2003.
 - [68] A. D. Nguyen *et al.*, "Y1 and Y5 Receptors Are Both Required for the Regulation of Food Intake and Energy Homeostasis in Mice," *PLoS One*, vol. 7, no. 6, p. e40191, Jun. 2012.
 - [69] M. M. Ollmann *et al.*, "Antagonism of Central Melanocortin Receptors in Vitro and in Vivo by Agouti-Related Protein," *Science (80-)*, vol. 278, no. 5335, pp. 135–138, Oct. 1997.
 - [70] M. A. Cowley *et al.*, "Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus," *Nature*, vol. 411, no. 6836, pp. 480–484, May 2001.
 - [71] Y. Aponte, D. Atasoy, and S. M. Sternson, "AGRP neurons are sufficient to orchestrate feeding behavior rapidly and without training," *Nat. Neurosci.*, vol. 14, no. 3, pp. 351–355, Mar. 2011.
 - [72] M. J. Krashes *et al.*, "Rapid, reversible activation of AgRP neurons drives feeding behavior in mice," *J. Clin. Invest.*, vol. 121, no. 4, pp. 1424–1428, Apr. 2011.
 - [73] C. Zhan *et al.*, "Acute and Long-Term Suppression of Feeding Behavior by POMC Neurons in the Brainstem and Hypothalamus, Respectively," *J. Neurosci.*, vol. 33, no. 8, pp. 3624–3632, Feb. 2013.
 - [74] H. Fenselau *et al.*, "A rapidly acting glutamatergic ARC→PVH satiety circuit postsynaptically regulated by α -MSH," *Nat. Neurosci.*, vol. 20, no. 1, pp. 42–51, Jan. 2017.
 - [75] R. V. Considine *et al.*, "Serum Immunoreactive-Leptin Concentrations in Normal-Weight and Obese Humans," *N. Engl. J. Med.*, vol. 334, no. 5, pp. 292–295, Feb. 1996.
 - [76] M. W. Schwartz, E. Peskind, M. Raskind, E. J. Boyko, and D. Porte, "Cerebrospinal fluid leptin levels: relationship to plasma levels and to adiposity in humans.," *Nat. Med.*, vol. 2, no. 5, pp. 589–93, May 1996.
 - [77] J. D. Bagdade, E. L. Bierman, and D. Porte, "The Significance of Basal Insulin Levels in the Evaluation of the Insulin Response to Glucose in Diabetic and Nondiabetic Subjects*," *J. Clin. Invest.*, vol. 46, no. 10, pp. 1549–1557, Oct. 1967.
 - [78] K. W. Williams *et al.*, "Segregation of Acute Leptin and Insulin Effects in Distinct Populations of Arcuate Proopiomelanocortin Neurons," *J. Neurosci.*, vol. 30, no. 7, pp. 2472–2479, Feb. 2010.
 - [79] D. Spanswick, M. A. Smith, S. Mirshamsi, V. H. Routh, and M. L. J. Ashford, "Insulin activates ATP-sensitive K⁺ channels in hypothalamic neurons of lean, but not obese rats," *Nat. Neurosci.*, vol. 3, no. 8, pp. 757–758, Aug. 2000.
 - [80] M. A. Cowley *et al.*, "The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis.," *Neuron*, vol. 37, no. 4, pp. 649–661, Feb. 2003.
 - [81] J. N. Betley *et al.*, "Neurons for hunger and thirst transmit a negative-valence teaching signal," *Nature*, vol. 521, no. 7551, pp. 180–185, May 2015.
 - [82] R. L. Batterham *et al.*, "Gut hormone PYY3-36 physiologically inhibits food intake," *Nature*, vol. 418, no. 6898, pp. 650–654, Aug. 2002.
 - [83] B. Y. H. Lam *et al.*, "Heterogeneity of hypothalamic pro-opiomelanocortin-expressing neurons revealed by single-cell RNA sequencing," *Mol. Metab.*, vol. 6, no. 5, pp. 383–392, May 2017.
 - [84] C. Mathis, T. H. Moran, and G. J. Schwartz, "Load-sensitive rat gastric vagal afferents encode volume but not gastric nutrients," *Am. J. Physiol. Integr. Comp. Physiol.*, vol. 274, no. 2, pp. R280–R286, Feb. 1998.
 - [85] H. J. Grill and M. R. Hayes, "Hindbrain neurons as an essential hub in the neuroanatomically distributed control of energy balance.," *Cell Metab.*, vol. 16, no. 3, pp. 296–309, Sep. 2012.
 - [86] M. R. Hayes *et al.*, "The common hepatic branch of the vagus is not required to mediate the glycemic and food intake suppressive effects of glucagon-like-peptide-1," *Am. J. Physiol. Integr. Comp. Physiol.*, vol. 301, no. 5, pp. R1479–R1485, Nov. 2011.
 - [87] C. R. Abbott *et al.*, "The inhibitory effects of peripheral administration of peptide YY(3-36) and glucagon-like peptide-1 on food intake are attenuated by ablation of the vagal-brainstem-hypothalamic pathway.," *Brain Res.*, vol. 1044, no. 1, pp. 127–31, May 2005.
 - [88] Y. Date *et al.*, "The role of the gastric afferent vagal nerve in ghrelin-induced feeding and

- growth hormone secretion in rats," *Gastroenterology*, vol. 123, no. 4, pp. 1120–1128, Oct. 2002.
- [89] C. Diepenbroek *et al.*, "Validation and characterization of a novel method for selective vagal deafferentation of the gut," *Am. J. Physiol. Liver Physiol.*, vol. 313, no. 4, pp. G342–G352, Oct. 2017.
- [90] G. P. Smith, C. Jerome, and R. Norgren, "Afferent axons in abdominal vagus mediate satiety effect of cholecystokinin in rats," *Am. J. Physiol. Integr. Comp. Physiol.*, vol. 249, no. 5, pp. R638–R641, Nov. 1985.
- [91] S. C. Cork, J. E. Richards, M. K. Holt, F. M. Gribble, F. Reimann, and S. Trapp, "Distribution and characterisation of Glucagon-like peptide-1 receptor expressing cells in the mouse brain.," *Mol. Metab.*, vol. 4, no. 10, pp. 718–31, Oct. 2015.
- [92] D. Stanić, P. Brumovsky, S. Fetissov, S. Shuster, H. Herzog, and T. Hökfelt, "Characterization of neuropeptide Y2 receptor protein expression in the mouse brain. I. Distribution in cell bodies and nerve terminals," *J. Comp. Neurol.*, vol. 499, no. 3, pp. 357–390, Nov. 2006.
- [93] D. R. Hill, N. J. Campbell, T. M. Shaw, and G. N. Woodruff, "Autoradiographic localization and biochemical characterization of peripheral type CCK receptors in rat CNS using highly selective nonpeptide CCK antagonists," *J. Neurosci.*, vol. 7, no. 9, pp. 2967–76, Sep. 1987.
- [94] H. J. Grill, M. W. Schwartz, J. M. Kaplan, J. S. Foxhall, J. Breininger, and D. G. Baskin, "Evidence That the Caudal Brainstem Is a Target for the Inhibitory Effect of Leptin on Food Intake," *Endocrinology*, vol. 143, no. 1, pp. 239–246, Jan. 2002.
- [95] J. M. Zigman, J. E. Jones, C. E. Lee, C. B. Saper, and J. K. Elmquist, "Expression of ghrelin receptor mRNA in the rat and the mouse brain," *J. Comp. Neurol.*, vol. 494, no. 3, pp. 528–548, Jan. 2006.
- [96] I. G. Halatchev and R. D. Cone, "Peripheral administration of PYY3–36 produces conditioned taste aversion in mice," *Cell Metab.*, vol. 1, no. 3, pp. 159–168, Mar. 2005.
- [97] H. Mönnikes, G. Lauer, and R. Arnold, "Peripheral administration of cholecystokinin activates c-fos expression in the locus coeruleus/subcoeruleus nucleus, dorsal vagal complex and paraventricular nucleus via capsaicin-sensitive vagal afferents and CCK-A receptors in the rat," *Brain Res.*, vol. 770, no. 1–2, pp. 277–288, Oct. 1997.
- [98] S. C. Benoit *et al.*, "A Novel Selective Melanocortin-4 Receptor Agonist Reduces Food Intake in Rats and Mice without Producing Aversive Consequences," *J. Neurosci.*, vol. 20, no. 9, pp. 3442–3448, May 2000.
- [99] C. F. Elias *et al.*, "Chemical characterization of leptin-activated neurons in the rat brain.," *J. Comp. Neurol.*, vol. 423, no. 2, pp. 261–81, Jul. 2000.
- [100] C. B. Lawrence, A. C. Snape, F. M.-H. Baudoin, and S. M. Luckman, "Acute Central Ghrelin and GH Secretagogues Induce Feeding and Activate Brain Appetite Centers," *Endocrinology*, vol. 143, no. 1, pp. 155–162, Jan. 2002.
- [101] M. K. Holt *et al.*, "Preproglucagon Neurons in the Nucleus of the Solitary Tract Are the Main Source of Brain GLP-1, Mediate Stress-Induced Hypophagia, and Limit Unusually Large Intakes of Food.," *Diabetes*, vol. 68, no. 1, pp. 21–33, 2019.
- [102] G. D'Agostino *et al.*, "Nucleus of the Solitary Tract Serotonin 5-HT_{2C} Receptors Modulate Food Intake," *Cell Metab.*, vol. 28, no. 4, pp. 619–630.e5, Oct. 2018.
- [103] H. Zheng *et al.*, "A potential role for hypothalamomedullary POMC projections in leptin-induced suppression of food intake," *Am. J. Physiol. Integr. Comp. Physiol.*, vol. 298, no. 3, pp. R720–R728, Mar. 2010.
- [104] J. Ciriello, J. C. McMurray, T. Babic, and C. V. R. de Oliveira, "Collateral axonal projections from hypothalamic hypocretin neurons to cardiovascular sites in nucleus ambiguus and nucleus tractus solitarius," *Brain Res.*, vol. 991, no. 1–2, pp. 133–141, Nov. 2003.
- [105] E. M. Parise *et al.*, "Evidence for the role of hindbrain orexin-1 receptors in the control of meal size," *Am. J. Physiol. Integr. Comp. Physiol.*, vol. 301, no. 6, pp. R1692–R1699, Dec. 2011.
- [106] C. B. de La Serre, Y. J. Kim, T. H. Moran, and S. Bi, "Dorsomedial hypothalamic NPY affects cholecystokinin-induced satiety via modulation of brain stem catecholamine neuronal signaling," *Am. J. Physiol. Integr. Comp. Physiol.*, vol. 311, no. 5, pp. R930–R939, Nov. 2016.
- [107] C. A. Campos, A. J. Bowen, M. W. Schwartz, and R. D. Palmiter, "Parabrachial CGRP Neurons Control Meal Termination," *Cell Metab.*, vol. 23, no. 5, pp. 811–820, May 2016.
- [108] G. D'Agostino *et al.*, "Appetite controlled by a cholecystokinin nucleus of the solitary tract to hypothalamus neurocircuit," *Elife*, vol. 5, Mar. 2016.
- [109] Y. Li, X. Wu, Y. Zhao, S. Chen, and C. Owyang, "Ghrelin acts on the dorsal vagal complex to stimulate pancreatic protein secretion," *Am. J. Physiol. Liver Physiol.*, vol. 290, no. 6, pp. G1350–G1358, Jun. 2006.

- [110] D. van der Kooy, "Area postrema: site where cholecystokinin acts to decrease food intake," *Brain Res.*, vol. 295, no. 2, pp. 345–347, Mar. 1984.
- [111] M. R. Hayes *et al.*, "Endogenous Leptin Signaling in the Caudal Nucleus Tractus Solitarius and Area Postrema Is Required for Energy Balance Regulation," *Cell Metab.*, vol. 11, no. 1, pp. 77–83, Jan. 2010.
- [112] J. E. Cox and A. Randich, "Enhancement of feeding suppression by PYY3-36 in rats with area postrema ablations," *Peptides*, vol. 25, no. 6, pp. 985–989, Jun. 2004.
- [113] E.-D. Baraboi, P. Smith, A. V. Ferguson, and D. Richard, "Lesions of area postrema and subfornical organ alter exendin-4-induced brain activation without preventing the hypophagic effect of the GLP-1 receptor agonist," *Am. J. Physiol. Integr. Comp. Physiol.*, vol. 298, no. 4, pp. R1098–R1110, Apr. 2010.
- [114] R. E. Shapiro and R. R. Miselis, "The central neural connections of the area postrema of the rat," *J. Comp. Neurol.*, vol. 234, no. 3, pp. 344–364, Apr. 1985.
- [115] K. N. Browning, S. Verheijden, and G. E. Boeckstaens, "The Vagus Nerve in Appetite Regulation, Mood, and Intestinal Inflammation," *Gastroenterology*, vol. 152, no. 4, pp. 730–744, Mar. 2017.
- [116] M. E. Carter, M. E. Soden, L. S. Zweifel, and R. D. Palmiter, "Genetic identification of a neural circuit that suppresses appetite," *Nature*, vol. 503, no. 7474, pp. 111–4, Nov. 2013.
- [117] R. A. Essner, A. G. Smith, A. A. Jamnik, A. R. Ryba, Z. D. Trutner, and M. E. Carter, "AgRP Neurons Can Increase Food Intake during Conditions of Appetite Suppression and Inhibit Anorexigenic Parabrachial Neurons," *J. Neurosci.*, vol. 37, no. 36, pp. 8678–8687, Sep. 2017.
- [118] C. W. Roman, V. A. Derkach, and R. D. Palmiter, "Genetically and functionally defined NTS to PBN brain circuits mediating anorexia," *Nat. Commun.*, vol. 7, no. 1, p. 11905, Sep. 2016.
- [119] R. D. Palmiter, "The Parabrachial Nucleus: CGRP Neurons Function as a General Alarm," *Trends Neurosci.*, vol. 41, no. 5, pp. 280–293, May 2018.
- [120] S. Mojsov, G. Heinrich, I. B. Wilson, M. Ravazzola, L. Orci, and J. F. Habener, "Preproglucagon gene expression in pancreas and intestine diversifies at the level of post-translational processing," *J. Biol. Chem.*, vol. 261, no. 25, pp. 11880–9, Sep. 1986.
- [121] P. J. Larsen, M. Tang-Christensen, J. J. Holst, and C. Orskov, "Distribution of glucagon-like peptide-1 and other preproglucagon-derived peptides in the rat hypothalamus and brainstem," *Neuroscience*, vol. 77, no. 1, pp. 257–70, Mar. 1997.
- [122] Y. Rouillé, S. Martin, and D. F. Steiner, "Differential Processing of Proglucagon by the Subtilisin-like Prohormone Convertases PC2 and PC3 to Generate either Glucagon or Glucagon-like Peptide," *J. Biol. Chem.*, vol. 270, no. 44, pp. 26488–26496, Nov. 1995.
- [123] C. Orskov, L. Rabenhøj, A. Wettergren, H. Kofod, and J. J. Holst, "Tissue and Plasma Concentrations of Amidated and Glycine-Extended Glucagon-Like Peptide I in Humans," *Diabetes*, vol. 43, no. 4, pp. 535–539, Apr. 1994.
- [124] C. Herrmann, R. Göke, G. Richter, H.-C. Fehmann, R. Arnold, and B. Göke, "Glucagon-Like Peptide-1 and Glucose-Dependent Insulin-Releasing Polypeptide Plasma Levels in Response to Nutrients," *Digestion*, vol. 56, no. 2, pp. 117–126, 1995.
- [125] R. Eissele *et al.*, "Glucagon-like peptide-1 cells in the gastrointestinal tract and pancreas of rat, pig and man," *Eur. J. Clin. Invest.*, vol. 22, no. 4, pp. 283–91, Apr. 1992.
- [126] V. Gorboulev *et al.*, "Na⁺-D-glucose Cotransporter SGLT1 is Pivotal for Intestinal Glucose Absorption and Glucose-Dependent Incretin Secretion," *Diabetes*, vol. 61, no. 1, pp. 187–196, Jan. 2012.
- [127] A. Alamshah *et al.*, "l-phenylalanine modulates gut hormone release and glucose tolerance, and suppresses food intake through the calcium-sensing receptor in rodents," *Int. J. Obes.*, vol. 41, no. 11, pp. 1693–1701, Nov. 2017.
- [128] H. V. Lin *et al.*, "GPR142 Controls Tryptophan-Induced Insulin and Incretin Hormone Secretion to Improve Glucose Metabolism," *PLoS One*, vol. 11, no. 6, p. e0157298, Jun. 2016.
- [129] C. P. Briscoe *et al.*, "The Orphan G Protein-coupled Receptor GPR40 Is Activated by Medium and Long Chain Fatty Acids," *J. Biol. Chem.*, vol. 278, no. 13, pp. 11303–11311, Mar. 2003.
- [130] A. S. Rocca and P. L. Brubaker, "Role of the Vagus Nerve in Mediating Proximal Nutrient-Induced Glucagon-Like Peptide-1 Secretion*," *Endocrinology*, vol. 140, no. 4, pp. 1687–1694, Apr. 1999.
- [131] C. F. Deacon, A. H. Johnsen, and J. J. Holst, "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.*, vol. 80, no. 3, pp. 952–957, Mar. 1995.
- [132] A. Plamboeck, J. J. Holst, R. D. Carr, and C. F. Deacon, "Neutral endopeptidase 24.11 and dipeptidyl peptidase IV are both mediators of the degradation of glucagon-like peptide 1 in the

- anaesthetised pig," *Diabetologia*, vol. 48, no. 9, pp. 1882–1890, Sep. 2005.
- [133] K. Hupe-Sodmann *et al.*, "Characterisation of the processing by human neutral endopeptidase 24.11 of GLP-1(7–36) amide and comparison of the substrate specificity of the enzyme for other glucagon-like peptides," *Regul. Pept.*, vol. 58, no. 3, pp. 149–156, Aug. 1995.
 - [134] C. F. Deacon, L. B. Knudsen, K. Madsen, F. C. Wiberg, O. Jacobsen, and J. J. Holst, "Dipeptidyl peptidase IV resistant analogues of glucagon-like peptide-1 which have extended metabolic stability and improved biological activity.," *Diabetologia*, vol. 41, no. 3, pp. 271–8, Mar. 1998.
 - [135] L. Hansen, C. F. Deacon, C. Ørskov, and J. J. Holst, "Glucagon-Like Peptide-1-(7–36)Amide Is Transformed to Glucagon-Like Peptide-1-(9–36)Amide by Dipeptidyl Peptidase IV in the Capillaries Supplying the L Cells of the Porcine Intestine 1," *Endocrinology*, vol. 140, no. 11, pp. 5356–5363, Nov. 1999.
 - [136] L. Hansen, B. Hartmann, T. Bisgaard, H. Mineo, P. N. Jørgensen, and J. J. Holst, "Somatostatin restrains the secretion of glucagon-like peptide-1 and -2 from isolated perfused porcine ileum," *Am. J. Physiol. Metab.*, vol. 278, no. 6, pp. E1010–E1018, Jun. 2000.
 - [137] C. F. Deacon, L. Pridal, L. Klarskov, M. Olesen, and J. J. Holst, "Glucagon-like peptide 1 undergoes differential tissue-specific metabolism in the anesthetized pig," *Am. J. Physiol. Metab.*, vol. 271, no. 3, pp. E458–E464, Sep. 1996.
 - [138] J. J. Holst, "The Physiology of Glucagon-like Peptide 1," *Physiol. Rev.*, vol. 87, no. 4, pp. 1409–1439, Oct. 2007.
 - [139] R. Mentlein, "Dipeptidyl-peptidase IV (CD26)--role in the inactivation of regulatory peptides.," *Regul. Pept.*, vol. 85, no. 1, pp. 9–24, Nov. 1999.
 - [140] J. J. Meier *et al.*, "Secretion, Degradation, and Elimination of Glucagon-Like Peptide 1 and Gastric Inhibitory Polypeptide in Patients with Chronic Renal Insufficiency and Healthy Control Subjects," *Diabetes*, vol. 53, no. 3, pp. 654–662, Mar. 2004.
 - [141] P. Richards *et al.*, "Identification and Characterization of GLP-1 Receptor-Expressing Cells Using a New Transgenic Mouse Model," *Diabetes*, vol. 63, no. 4, pp. 1224–1233, Apr. 2014.
 - [142] C. Pyke *et al.*, "GLP-1 Receptor Localization in Monkey and Human Tissue: Novel Distribution Revealed With Extensively Validated Monoclonal Antibody," *Endocrinology*, vol. 155, no. 4, pp. 1280–1290, Apr. 2014.
 - [143] C. Orskov, J. J. Holst, and O. V Nielsen, "Effect of truncated glucagon-like peptide-1 [proglucagon-(78-107) amide] on endocrine secretion from pig pancreas, antrum, and nonantral stomach.," *Endocrinology*, vol. 123, no. 4, pp. 2009–13, Oct. 1988.
 - [144] L. Farilla *et al.*, "Glucagon-Like Peptide 1 Inhibits Cell Apoptosis and Improves Glucose Responsiveness of Freshly Isolated Human Islets," *Endocrinology*, vol. 144, no. 12, pp. 5149–5158, Dec. 2003.
 - [145] G. Xu, D. A. Stoffers, J. F. Habener, and S. Bonner-Weir, "Exendin-4 stimulates both beta-cell replication and neogenesis, resulting in increased beta-cell mass and improved glucose tolerance in diabetic rats," *Diabetes*, vol. 48, no. 12, pp. 2270–2276, Dec. 1999.
 - [146] A. Wettergren, B. Schjoldager, P. E. Mortensen, J. Myhre, J. Christiansen, and J. J. Holst, "Truncated GLP-1 (proglucagon 78-107-amide) inhibits gastric and pancreatic functions in man.," *Dig. Dis. Sci.*, vol. 38, no. 4, pp. 665–73, Apr. 1993.
 - [147] A. Flint, A. Raben, A. Astrup, and J. J. Holst, "Glucagon-like peptide 1 promotes satiety and suppresses energy intake in humans.," *J. Clin. Invest.*, vol. 101, no. 3, pp. 515–520, Feb. 1998.
 - [148] M. J. During *et al.*, "Glucagon-like peptide-1 receptor is involved in learning and neuroprotection," *Nat. Med.*, vol. 9, no. 9, pp. 1173–1179, Sep. 2003.
 - [149] A. K. Bose, M. M. Mocanu, R. D. Carr, C. L. Brand, and D. M. Yellon, "Glucagon-like Peptide 1 Can Directly Protect the Heart Against Ischemia/Reperfusion Injury," *Diabetes*, vol. 54, no. 1, pp. 146–151, Jan. 2005.
 - [150] C. Yamada *et al.*, "The Murine Glucagon-Like Peptide-1 Receptor Is Essential for Control of Bone Resorption," *Endocrinology*, vol. 149, no. 2, pp. 574–579, Feb. 2008.
 - [151] J.-P. Gutzwiller *et al.*, "Glucagon-Like Peptide 1 Induces Natriuresis in Healthy Subjects and in Insulin-Resistant Obese Men," *J. Clin. Endocrinol. Metab.*, vol. 89, no. 6, pp. 3055–3061, Jun. 2004.
 - [152] C. Orskov, S. S. Poulsen, M. Møller, and J. J. Holst, "Glucagon-like peptide I receptors in the subfornical organ and the area postrema are accessible to circulating glucagon-like peptide I.," *Diabetes*, vol. 45, no. 6, pp. 832–5, Jun. 1996.
 - [153] J. Schirra *et al.*, "Endogenous glucagon-like peptide 1 controls endocrine pancreatic secretion and antro-pyloro-duodenal motility in humans.," *Gut*, vol. 55, no. 2, pp. 243–51, Feb. 2006.

- [154] M. J. Bak *et al.*, "Specificity and sensitivity of commercially available assays for glucagon-like peptide-1 (GLP-1): implications for GLP-1 measurements in clinical studies," *Diabetes, Obes. Metab.*, vol. 16, no. 11, pp. 1155–1164, Nov. 2014.
- [155] D. J. Hodson *et al.*, "Lipotoxicity disrupts incretin-regulated human β cell connectivity," *J. Clin. Invest.*, vol. 123, no. 10, pp. 4182–4194, Oct. 2013.
- [156] B. J. Lamont, Y. Li, E. Kwan, T. J. Brown, H. Gaisano, and D. J. Drucker, "Pancreatic GLP-1 receptor activation is sufficient for incretin control of glucose metabolism in mice," *J. Clin. Invest.*, vol. 122, no. 1, pp. 388–402, Jan. 2012.
- [157] H.-R. Berthoud and W. L. Neuhuber, "Functional and chemical anatomy of the afferent vagal system," *Auton. Neurosci.*, vol. 85, no. 1–3, pp. 1–17, Dec. 2000.
- [158] A. Nakagawa *et al.*, "Receptor gene expression of glucagon-like peptide-1, but not glucose-dependent insulinotropic polypeptide, in rat nodose ganglion cells," *Auton. Neurosci.*, vol. 110, no. 1, pp. 36–43, Jan. 2004.
- [159] L. Rinaman, J. P. Card, J. S. Schwaber, and R. R. Miselis, "Ultrastructural demonstration of a gastric monosynaptic vagal circuit in the nucleus of the solitary tract in rat," *J. Neurosci.*, vol. 9, no. 6, pp. 1985–96, Jun. 1989.
- [160] M. R. Hayes, E. G. Mietlicki-Baase, S. E. Kanoski, and B. C. De Jonghe, "Incretins and Amylin: Neuroendocrine Communication Between the Gut, Pancreas, and Brain in Control of Food Intake and Blood Glucose," *Annu. Rev. Nutr.*, vol. 34, no. 1, pp. 237–260, Jul. 2014.
- [161] H.-R. Berthoud and T. L. Powley, "Morphology and distribution of efferent vagal innervation of rat pancreas as revealed with anterograde transport of Dil," *Brain Res.*, vol. 553, no. 2, pp. 336–341, Jul. 1991.
- [162] J.-P. Krieger, M. Arnold, K. G. Pettersen, P. Lossel, W. Langhans, and S. J. Lee, "Knockdown of GLP-1 receptors in vagal afferents affects normal food intake and glycemia," *Diabetes*, p. db150973, Oct. 2015.
- [163] E. M. Varin *et al.*, "Distinct Neural Sites of GLP-1R Expression Mediate Physiological versus Pharmacological Control of Incretin Action," *Cell Rep.*, vol. 27, no. 11, pp. 3371–3384.e3, Jun. 2019.
- [164] E. P. Smith *et al.*, "The Role of β Cell Glucagon-like Peptide-1 Signaling in Glucose Regulation and Response to Diabetes Drugs," *Cell Metab.*, vol. 19, no. 6, pp. 1050–1057, Jun. 2014.
- [165] M. Nishizawa, H. Nakabayashi, K. Uehara, A. Nakagawa, K. Uchida, and D. Koya, "Intraportal GLP-1 stimulates insulin secretion predominantly through the hepatoportal-pancreatic vagal reflex pathways," *Am. J. Physiol. Metab.*, vol. 305, no. 3, pp. E376–E387, Aug. 2013.
- [166] K. Fujiwara *et al.*, "Intraportal administration of DPP-IV inhibitor regulates insulin secretion and food intake mediated by the hepatic vagal afferent nerve in rats," *J. Neurochem.*, vol. 121, no. 1, pp. 66–76, Apr. 2012.
- [167] Y. Nie *et al.*, "Regulation of pancreatic PC1 and PC2 associated with increased glucagon-like peptide 1 in diabetic rats," *J. Clin. Invest.*, vol. 105, no. 7, pp. 955–965, Apr. 2000.
- [168] G. Kilimnik, A. Kim, D. F. Steiner, T. C. Friedman, and M. Hara, "Intracistern production of GLP-1 by activation of prohormone convertase 1/3 in pancreatic α -cells in mouse models of β -cell regeneration," *Islets*, vol. 2, no. 3, pp. 149–155, May 2010.
- [169] A. M. K. Hansen *et al.*, "Upregulation of alpha cell glucagon-like peptide 1 (GLP-1) in *Psammomys obesus*—an adaptive response to hyperglycaemia?," *Diabetologia*, vol. 54, no. 6, pp. 1379–1387, Jun. 2011.
- [170] H. Ellingsgaard *et al.*, "Interleukin-6 enhances insulin secretion by increasing glucagon-like peptide-1 secretion from L cells and alpha cells," *Nat. Med.*, vol. 17, no. 11, pp. 1481–1489, Nov. 2011.
- [171] S. Traub *et al.*, "Pancreatic α Cell-Derived Glucagon-Related Peptides Are Required for β Cell Adaptation and Glucose Homeostasis," *Cell Rep.*, vol. 18, no. 13, pp. 3192–3203, Mar. 2017.
- [172] P. Marchetti *et al.*, "A local glucagon-like peptide 1 (GLP-1) system in human pancreatic islets," *Diabetologia*, vol. 55, no. 12, pp. 3262–3272, Dec. 2012.
- [173] D. Bosco *et al.*, "Unique Arrangement of α - and β -Cells in Human Islets of Langerhans," *Diabetes*, vol. 59, no. 5, pp. 1202–1210, May 2010.
- [174] O. Cabrera, D. M. Berman, N. S. Kenyon, C. Ricordi, P.-O. Berggren, and A. Caicedo, "The unique cytoarchitecture of human pancreatic islets has implications for islet cell function," *Proc. Natl. Acad. Sci.*, vol. 103, no. 7, pp. 2334–2339, Feb. 2006.
- [175] R. Rodriguez-Diaz *et al.*, "Alpha cells secrete acetylcholine as a non-neuronal paracrine signal priming beta cell function in humans," *Nat. Med.*, vol. 17, no. 7, pp. 888–892, Jul. 2011.
- [176] A. P. Chambers *et al.*, "The Role of Pancreatic Preproglucagon in Glucose Homeostasis in Mice," *Cell Metab.*, vol. 25, no. 4, pp. 927–934.e3, Apr. 2017.

- [177] B. Svendsen *et al.*, "Insulin Secretion Depends on Intra-islet Glucagon Signaling," *Cell Rep.*, vol. 25, no. 5, pp. 1127–1134.e2, Oct. 2018.
- [178] K. D. Galsgaard *et al.*, "Disruption of glucagon receptor signaling causes hyperaminoacidemia exposing a possible liver-alpha-cell axis," *Am. J. Physiol. Metab.*, vol. 314, no. 1, pp. E93–E103, Jan. 2018.
- [179] A. Secher *et al.*, "The arcuate nucleus mediates GLP-1 receptor agonist liraglutide-dependent weight loss," *J. Clin. Invest.*, vol. 124, no. 10, pp. 4473–4488, Oct. 2014.
- [180] S. Sisley, R. Gutierrez-Aguilar, M. Scott, D. A. D'Alessio, D. A. Sandoval, and R. J. Seeley, "Neuronal GLP1R mediates liraglutide's anorectic but not glucose-lowering effect.," *J. Clin. Invest.*, vol. 124, no. 6, pp. 2456–63, Jun. 2014.
- [181] B. Laferrere *et al.*, "Incretin Levels and Effect Are Markedly Enhanced 1 Month After Roux-en-Y Gastric Bypass Surgery in Obese Patients With Type 2 Diabetes," *Diabetes Care*, vol. 30, no. 7, pp. 1709–1716, Jul. 2007.
- [182] C. W. le Roux *et al.*, "Gut Hormone Profiles Following Bariatric Surgery Favor an Anorectic State, Facilitate Weight Loss, and Improve Metabolic Parameters," *Ann. Surg.*, vol. 243, no. 1, pp. 108–114, Jan. 2006.
- [183] D. Garibay *et al.*, "β-Cell Glucagon-Like Peptide-1 Receptor Contributes to Improved Glucose Tolerance After Vertical Sleeve Gastrectomy," *Endocrinology*, vol. 157, no. 9, pp. 3405–3409, Sep. 2016.
- [184] D. L. Williams, D. G. Baskin, and M. W. Schwartz, "Leptin regulation of the anorectic response to glucagon-like peptide-1 receptor stimulation.," *Diabetes*, vol. 55, no. 12, pp. 3387–93, Dec. 2006.
- [185] P. K. Chelikani, A. C. Haver, and R. D. Reidelberger, "Intravenous infusion of glucagon-like peptide-1 potently inhibits food intake, sham feeding, and gastric emptying in rats.," *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, vol. 288, no. 6, pp. R1695–706, Jun. 2005.
- [186] M. D. Turton *et al.*, "A role for glucagon-like peptide-1 in the central regulation of feeding.," *Nature*, vol. 379, no. 6560, pp. 69–72, Jan. 1996.
- [187] D. L. Williams, D. G. Baskin, and M. W. Schwartz, "Evidence that intestinal glucagon-like peptide-1 plays a physiological role in satiety.," *Endocrinology*, vol. 150, no. 4, pp. 1680–7, Apr. 2009.
- [188] L. L. Baggio, Q. Huang, T. J. Brown, and D. J. Drucker, "A recombinant human glucagon-like peptide (GLP)-1-albumin protein (albugon) mimics peptidergic activation of GLP-1 receptor-dependent pathways coupled with satiety, gastrointestinal motility, and glucose homeostasis.," *Diabetes*, vol. 53, no. 9, pp. 2492–500, Sep. 2004.
- [189] L. L. Baggio, Q. Huang, X. Cao, and D. J. Drucker, "An albumin-exendin-4 conjugate engages central and peripheral circuits regulating murine energy and glucose homeostasis.," *Gastroenterology*, vol. 134, no. 4, pp. 1137–47, Apr. 2008.
- [190] T. Talsania, Y. Anini, S. Siu, D. J. Drucker, and P. L. Brubaker, "Peripheral exendin-4 and peptide YY(3-36) synergistically reduce food intake through different mechanisms in mice.," *Endocrinology*, vol. 146, no. 9, pp. 3748–56, Sep. 2005.
- [191] E. B. Rüttimann, M. Arnold, J. J. Hillebrand, N. Geary, and W. Langhans, "Intrameal hepatic portal and intraperitoneal infusions of glucagon-like peptide-1 reduce spontaneous meal size in the rat via different mechanisms.," *Endocrinology*, vol. 150, no. 3, pp. 1174–81, Mar. 2009.
- [192] J. Zhang and R. C. Ritter, "Circulating GLP-1 and CCK-8 reduce food intake by capsaicin-insensitive, nonvagal mechanisms.," *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, vol. 302, no. 2, pp. R264–73, Jan. 2012.
- [193] S. E. Kanoski, M. R. Hayes, and K. P. Skibicka, "GLP-1 and weight loss: unraveling the diverse neural circuitry," *Am. J. Physiol. Integr. Comp. Physiol.*, vol. 310, no. 10, pp. R885–R895, May 2016.
- [194] A. J. Kastin, V. Akerstrom, and W. Pan, "Interactions of Glucagon-Like Peptide-1 (GLP-1) with the Blood-Brain Barrier," *J. Mol. Neurosci.*, vol. 18, no. 1–2, pp. 07–13, 2002.
- [195] S. M. Kanse, B. Kreymann, M. A. Ghatei, and S. R. Bloom, "Identification and characterization of glucagon-like peptide-1 7-36 amide-binding sites in the rat brain and lung.," *FEBS Lett.*, vol. 241, no. 1–2, pp. 209–12, Dec. 1988.
- [196] S. E. Kanoski, S. M. Fortin, M. Arnold, H. J. Grill, and M. R. Hayes, "Peripheral and central GLP-1 receptor populations mediate the anorectic effects of peripherally administered GLP-1 receptor agonists, liraglutide and exendin-4.," *Endocrinology*, vol. 152, no. 8, pp. 3103–12, Aug. 2011.
- [197] C. B. Jensen, C. Pyke, M. G. Rasch, A. B. Dahl, L. B. Knudsen, and A. Secher, "Characterization of the Glucagonlike Peptide-1 Receptor in Male Mouse Brain Using a Novel

- Antibody and In Situ Hybridization," *Endocrinology*, vol. 159, no. 2, pp. 665–675, Feb. 2018.
- [198] M. R. Hayes, L. Bradley, and H. J. Grill, "Endogenous hindbrain glucagon-like peptide-1 receptor activation contributes to the control of food intake by mediating gastric satiation signaling," *Endocrinology*, vol. 150, no. 6, pp. 2654–9, Jun. 2009.
 - [199] M. R. Hayes, K. P. Skibicka, and H. J. Grill, "Caudal brainstem processing is sufficient for behavioral, sympathetic, and parasympathetic responses driven by peripheral and hindbrain glucagon-like-peptide-1 receptor stimulation," *Endocrinology*, vol. 149, no. 8, pp. 4059–68, Aug. 2008.
 - [200] K. Hisadome, F. Reimann, F. M. Gribble, and S. Trapp, "Leptin directly depolarizes preproglucagon neurons in the nucleus tractus solitarius: electrical properties of glucagon-like Peptide 1 neurons," *Diabetes*, vol. 59, no. 8, pp. 1890–8, Aug. 2010.
 - [201] J. P. Card *et al.*, "GLP-1 neurons form a local synaptic circuit within the rodent nucleus of the solitary tract," *J. Comp. Neurol.*, vol. 526, no. 14, pp. 2149–2164, Oct. 2018.
 - [202] S. L. Jin, V. K. Han, J. G. Simmons, A. C. Towle, J. M. Lauder, and P. K. Lund, "Distribution of glucagonlike peptide I (GLP-I), glucagon, and glicentin in the rat brain: an immunocytochemical study," *J. Comp. Neurol.*, vol. 271, no. 4, pp. 519–32, May 1988.
 - [203] J. G. Barrera, K. R. Jones, J. P. Herman, D. A. D'Alessio, S. C. Woods, and R. J. Seeley, "Hyperphagia and increased fat accumulation in two models of chronic CNS glucagon-like peptide-1 loss of function," *J. Neurosci.*, vol. 31, no. 10, pp. 3904–13, Mar. 2011.
 - [204] I. J. Llewellyn-Smith, F. Reimann, F. M. Gribble, and S. Trapp, "Preproglucagon neurons project widely to autonomic control areas in the mouse brain," *Neuroscience*, vol. 180, pp. 111–21, Apr. 2011.
 - [205] I. J. Llewellyn-Smith, G. J. E. Gnanamanickam, F. Reimann, F. M. Gribble, and S. Trapp, "Preproglucagon (PPG) neurons innervate neurochemically identified autonomic neurons in the mouse brainstem," *Neuroscience*, vol. 229, pp. 130–43, Jan. 2013.
 - [206] I. J. Llewellyn-Smith, N. Marina, R. N. Manton, F. Reimann, F. M. Gribble, and S. Trapp, "Spinally projecting preproglucagon axons preferentially innervate sympathetic preganglionic neurons," *Neuroscience*, vol. 284, pp. 872–87, Jan. 2015.
 - [207] K. Hisadome, F. Reimann, F. M. Gribble, and S. Trapp, "CCK stimulation of GLP-1 neurons involves $\alpha 1$ -adrenoceptor-mediated increase in glutamatergic synaptic inputs," *Diabetes*, vol. 60, no. 11, pp. 2701–9, Nov. 2011.
 - [208] N. Vrang, C. B. Phifer, M. M. Corkern, and H.-R. Berthoud, "Gastric distension induces c-Fos in medullary GLP-1/2-containing neurons," *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, vol. 285, no. 2, pp. R470–8, Aug. 2003.
 - [209] D. A. Sandoval, D. Bagnol, S. C. Woods, D. A. D'Alessio, and R. J. Seeley, "Arcuate glucagon-like peptide 1 receptors regulate glucose homeostasis but not food intake," *Diabetes*, vol. 57, no. 8, pp. 2046–54, Aug. 2008.
 - [210] M. A. Burmeister *et al.*, "The Hypothalamic Glucagon-Like Peptide 1 Receptor Is Sufficient but Not Necessary for the Regulation of Energy Balance and Glucose Homeostasis in Mice," *Diabetes*, vol. 66, no. 2, pp. 372–384, Feb. 2017.
 - [211] D. Beiroa *et al.*, "GLP-1 agonism stimulates brown adipose tissue thermogenesis and browning through hypothalamic AMPK," *Diabetes*, vol. 63, no. 10, pp. 3346–58, Oct. 2014.
 - [212] K. Katsurada *et al.*, "Endogenous GLP-1 acts on paraventricular nucleus to suppress feeding: projection from nucleus tractus solitarius and activation of corticotropin-releasing hormone, nesfatin-1 and oxytocin neurons," *Biochem. Biophys. Res. Commun.*, vol. 451, no. 2, pp. 276–81, Aug. 2014.
 - [213] R. R. Schick, J. P. Zimmermann, T. vom Walde, and V. Schusdziarra, "Peptides that regulate food intake: glucagon-like peptide 1-(7-36) amide acts at lateral and medial hypothalamic sites to suppress feeding in rats," *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, vol. 284, no. 6, pp. R1427–35, Jun. 2003.
 - [214] M. A. Burmeister *et al.*, "The glucagon-like peptide-1 receptor in the ventromedial hypothalamus reduces short-term food intake in male mice by regulating nutrient sensor activity," *Am. J. Physiol. Endocrinol. Metab.*, vol. 313, no. 6, pp. E651–E662, 2017.
 - [215] A. L. Alhadeff, L. E. Rupprecht, and M. R. Hayes, "GLP-1 neurons in the nucleus of the solitary tract project directly to the ventral tegmental area and nucleus accumbens to control for food intake," *Endocrinology*, vol. 153, no. 2, pp. 647–58, Feb. 2012.
 - [216] A. M. Dossat, R. Diaz, L. Gallo, A. Panagos, K. Kay, and D. L. Williams, "Nucleus accumbens GLP-1 receptors influence meal size and palatability," *Am. J. Physiol. Endocrinol. Metab.*, vol. 304, no. 12, pp. E1314–20, Jun. 2013.
 - [217] A. M. Dossat, N. Lilly, K. Kay, and D. L. Williams, "Glucagon-like peptide 1 receptors in

- nucleus accumbens affect food intake.," *J. Neurosci.*, vol. 31, no. 41, pp. 14453–7, Oct. 2011.
- [218] R. H. Shirazi, S. L. Dickson, and K. P. Skibicka, "Gut peptide GLP-1 and its analogue, Exendin-4, decrease alcohol intake and reward.," *PLoS One*, vol. 8, no. 4, p. e61965, 2013.
- [219] S. L. Dickson, R. H. Shirazi, C. Hansson, F. Bergquist, H. Nissbrandt, and K. P. Skibicka, "The glucagon-like peptide 1 (GLP-1) analogue, exendin-4, decreases the rewarding value of food: a new role for mesolimbic GLP-1 receptors.," *J. Neurosci.*, vol. 32, no. 14, pp. 4812–20, Apr. 2012.
- [220] G. Sørensen *et al.*, "The glucagon-like peptide 1 (GLP-1) receptor agonist exendin-4 reduces cocaine self-administration in mice.," *Physiol. Behav.*, vol. 149, pp. 262–8, Oct. 2015.
- [221] T. M. Hsu, J. D. Hahn, V. R. Konanur, A. Lam, and S. E. Kanoski, "Hippocampal GLP-1 receptors influence food intake, meal size, and effort-based responding for food through volume transmission.," *Neuropsychopharmacology*, vol. 40, no. 2, pp. 327–37, Jan. 2015.
- [222] S. J. Terrill *et al.*, "Role of lateral septum glucagon-like peptide 1 receptors in food intake," *Am. J. Physiol. Integr. Comp. Physiol.*, vol. 311, no. 1, pp. R124–R132, Jul. 2016.
- [223] D. J. Reiner *et al.*, "Glucagon-Like Peptide-1 Receptor Signaling in the Lateral Dorsal Tegmental Nucleus Regulates Energy Balance.," *Neuropsychopharmacology*, vol. 43, no. 3, pp. 627–637, 2018.
- [224] G. M. Singewald, A. Rjabokon, N. Singewald, and K. Ebner, "The modulatory role of the lateral septum on neuroendocrine and behavioral stress responses.," *Neuropsychopharmacology*, vol. 36, no. 4, pp. 793–804, Mar. 2011.
- [225] G. C. Sartor and G. S. Aston-Jones, "A septal-hypothalamic pathway drives orexin neurons, which is necessary for conditioned cocaine preference.," *J. Neurosci.*, vol. 32, no. 13, pp. 4623–31, Mar. 2012.
- [226] T. E. Anthony, N. Dee, A. Bernard, W. Lerchner, N. Heintz, and D. J. Anderson, "Control of Stress-Induced Persistent Anxiety by an Extra-Amygdala Septohypothalamic Circuit," *Cell*, vol. 156, no. 3, pp. 522–536, Jan. 2014.
- [227] S. J. Terrill, C. B. Maske, and D. L. Williams, "Endogenous GLP-1 in lateral septum contributes to stress-induced hypophagia.," *Physiol. Behav.*, vol. 192, pp. 17–22, 2018.
- [228] P. Sweeney and Y. Yang, "An excitatory ventral hippocampus to lateral septum circuit that suppresses feeding.," *Nat. Commun.*, vol. 6, p. 10188, Dec. 2015.
- [229] P. Sweeney and Y. Yang, "An Inhibitory Septum to Lateral Hypothalamus Circuit That Suppresses Feeding.," *J. Neurosci.*, vol. 36, no. 44, pp. 11185–11195, 2016.
- [230] B. Åhrén, M. Landin-Olsson, P.-A. Jansson, M. Svensson, D. Holmes, and A. Schweizer, "Inhibition of Dipeptidyl Peptidase-4 Reduces Glycemia, Sustains Insulin Levels, and Reduces Glucagon Levels in Type 2 Diabetes," *J. Clin. Endocrinol. Metab.*, vol. 89, no. 5, pp. 2078–2084, May 2004.
- [231] B. Omar and B. Åhren, "Pleiotropic Mechanisms for the Glucose-Lowering Action of DPP-4 Inhibitors," *Diabetes*, vol. 63, no. 7, pp. 2196–2202, Jul. 2014.
- [232] E. S. Andersen, C. F. Deacon, and J. J. Holst, "Do we know the true mechanism of action of the DPP-4 inhibitors?," *Diabetes. Obes. Metab.*, vol. 20, no. 1, pp. 34–41, Dec. 2018.
- [233] V. R. Aroda *et al.*, "Efficacy of GLP-1 Receptor Agonists and DPP-4 Inhibitors: Meta-Analysis and Systematic Review," *Clin. Ther.*, vol. 34, no. 6, pp. 1247–1258.e22, Jun. 2012.
- [234] C. F. Deacon, "A review of dipeptidyl peptidase-4 inhibitors. Hot topics from randomized controlled trials," *Diabetes. Obes. Metab.*, vol. 20, pp. 34–46, Feb. 2018.
- [235] M. Yu *et al.*, "Battle of GLP-1 delivery technologies," *Adv. Drug Deliv. Rev.*, vol. 130, pp. 113–130, May 2018.
- [236] J. Lau *et al.*, "Discovery of the Once-Weekly Glucagon-Like Peptide-1 (GLP-1) Analogue Semaglutide," *J. Med. Chem.*, vol. 58, no. 18, pp. 7370–7380, Sep. 2015.
- [237] E. Sebkova *et al.*, "Taspoglutide, an Analog of Human Glucagon-Like Peptide-1 with Enhanced Stability and in Vivo Potency," *Endocrinology*, vol. 151, no. 6, pp. 2474–2482, Jun. 2010.
- [238] L. Simonsen, J. J. Holst, and C. F. Deacon, "Exendin-4, but not glucagon-like peptide-1, is cleared exclusively by glomerular filtration in anaesthetised pigs," *Diabetologia*, vol. 49, no. 4, pp. 706–712, Apr. 2006.
- [239] K. Copley, K. McCowen, R. Hiles, L. L. Nielsen, A. Young, and D. G. Parkes, "Investigation of exenatide elimination and its in vivo and in vitro degradation.," *Curr. Drug Metab.*, vol. 7, no. 4, pp. 367–74, May 2006.
- [240] C. M. Edwards *et al.*, "Exendin-4 reduces fasting and postprandial glucose and decreases energy intake in healthy volunteers.," *Am. J. Physiol. Endocrinol. Metab.*, vol. 281, no. 1, pp. E155–61, Jul. 2001.

- [241] B. Cirincione and D. E. Mager, "Population pharmacokinetics of exenatide," *Br. J. Clin. Pharmacol.*, vol. 83, no. 3, pp. 517–526, Mar. 2017.
- [242] T. MORETTO *et al.*, "Efficacy and tolerability of exenatide monotherapy over 24 weeks in antidiabetic drug—naïve patients with type 2 diabetes: A randomized, double-blind, placebo-controlled, parallel-group study," *Clin. Ther.*, vol. 30, no. 8, pp. 1448–1460, Aug. 2008.
- [243] T. Blevins *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.*, vol. 96, no. 5, pp. 1301–1310, May 2011.
- [244] J. Rosenstock *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*, vol. 36, no. 10, pp. 2945–2951, Oct. 2013.
- [245] V. A. Fonseca, R. Alvarado-Ruiz, D. Raccach, G. Boka, P. Miossec, and J. E. Gerich, "Efficacy and Safety of the Once-Daily GLP-1 Receptor Agonist Lixisenatide in Monotherapy: A randomized, double-blind, placebo-controlled trial in patients with type 2 diabetes (GetGoal-Mono)," *Diabetes Care*, vol. 35, no. 6, pp. 1225–1231, Jun. 2012.
- [246] T. M. Frederiksen *et al.*, "Oligomerization of a Glucagon-like Peptide 1 Analog: Bridging Experiment and Simulations," *Biophys. J.*, vol. 109, no. 6, pp. 1202–1213, Sep. 2015.
- [247] E. Watson, D. M. Jonker, L. V. Jacobsen, and S. H. Ingwersen, "Population Pharmacokinetics of Liraglutide, a Once-Daily Human Glucagon-Like Peptide-1 Analog, in Healthy Volunteers and Subjects With Type 2 Diabetes, and Comparison to Twice-Daily Exenatide," *J. Clin. Pharmacol.*, vol. 50, no. 8, pp. 886–894, Aug. 2010.
- [248] A. Plum, L. B. Jensen, and J. B. Kristensen, "In Vitro Protein Binding of Liraglutide in Human Plasma Determined by Reiterated Stepwise Equilibrium Dialysis," *J. Pharm. Sci.*, vol. 102, no. 8, pp. 2882–2888, Aug. 2013.
- [249] M. Malm-Erfjelt *et al.*, "Metabolism and Excretion of the Once-Daily Human Glucagon-Like Peptide-1 Analog Liraglutide in Healthy Male Subjects and Its In Vitro Degradation by Dipeptidyl Peptidase IV and Neutral Endopeptidase," *Drug Metab. Dispos.*, vol. 38, no. 11, pp. 1944–1953, Nov. 2010.
- [250] A. Garber *et al.*, "Liraglutide versus glimepiride monotherapy for type 2 diabetes (LEAD-3 Mono): a randomised, 52-week, phase III, double-blind, parallel-treatment trial," *Lancet*, vol. 373, no. 9662, pp. 473–481, Feb. 2009.
- [251] C. Sorli *et al.*, "Efficacy and safety of once-weekly semaglutide monotherapy versus placebo in patients with type 2 diabetes (SUSTAIN 1): a double-blind, randomised, placebo-controlled, parallel-group, multinational, multicentre phase 3a trial," *Lancet Diabetes Endocrinol.*, vol. 5, no. 4, pp. 251–260, Apr. 2017.
- [252] J. B. Buse *et al.*, "Liraglutide once a day versus exenatide twice a day for type 2 diabetes: a 26-week randomised, parallel-group, multinational, open-label trial (LEAD-6)," *Lancet*, vol. 374, no. 9683, pp. 39–47, Jul. 2009.
- [253] X. Pi-Sunyer *et al.*, "A Randomized, Controlled Trial of 3.0 mg of Liraglutide in Weight Management," *N. Engl. J. Med.*, vol. 373, no. 1, pp. 11–22, Jul. 2015.
- [254] A. Mehta, S. P. Marso, and I. J. Neeland, "Liraglutide for weight management: a critical review of the evidence," *Obes. Sci. Pract.*, vol. 3, no. 1, pp. 3–14, Mar. 2017.
- [255] S. P. Marso *et al.*, "Liraglutide and Cardiovascular Outcomes in Type 2 Diabetes," *N. Engl. J. Med.*, vol. 375, no. 4, pp. 311–322, Jul. 2016.
- [256] S. Verma *et al.*, "Effects of Liraglutide on Cardiovascular Outcomes in Patients With Type 2 Diabetes Mellitus With or Without History of Myocardial Infarction or Stroke," *Circulation*, vol. 138, no. 25, pp. 2884–2894, Dec. 2018.
- [257] S. P. Marso *et al.*, "Semaglutide and Cardiovascular Outcomes in Patients with Type 2 Diabetes," *N. Engl. J. Med.*, vol. 375, no. 19, pp. 1834–1844, Nov. 2016.
- [258] P. M. O'Neil *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*, vol. 392, no. 10148, pp. 637–649, Aug. 2018.
- [259] L. B. Knudsen and J. Lau, "The Discovery and Development of Liraglutide and Semaglutide," *Front. Endocrinol. (Lausanne)*, vol. 10, Apr. 2019.
- [260] M. Davies, T. R. Pieber, M.-L. Hartoft-Nielsen, O. K. H. Hansen, S. Jabbour, and J. Rosenstock, "Effect of Oral Semaglutide Compared With Placebo and Subcutaneous Semaglutide on Glycemic Control in Patients With Type 2 Diabetes," *JAMA*, vol. 318, no. 15, p. 1460, Oct. 2017.
- [261] V. R. ARODA *et al.*, "Effect and Safety of Oral Semaglutide Monotherapy in Type 2 Diabetes—

- PIONEER 1 Trial," *Diabetes*, vol. 67, no. Supplement 1, pp. 2-LB, May 2018.
- [262] J. Rosenstock *et al.*, "Effect of Additional Oral Semaglutide vs Sitagliptin on Glycated Hemoglobin in Adults With Type 2 Diabetes Uncontrolled With Metformin Alone or With Sulfonylurea," *JAMA*, vol. 321, no. 15, p. 1466, Apr. 2019.
 - [263] J. E. Matthews *et al.*, "Pharmacodynamics, Pharmacokinetics, Safety, and Tolerability of Albiglutide, a Long-Acting Glucagon-Like Peptide-1 Mimetic, in Patients with Type 2 Diabetes," *J. Clin. Endocrinol. Metab.*, vol. 93, no. 12, pp. 4810–4817, Dec. 2008.
 - [264] W. Glaesner *et al.*, "Engineering and characterization of the long-acting glucagon-like peptide-1 analogue LY2189265, an Fc fusion protein," *Diabetes. Metab. Res. Rev.*, vol. 26, no. 4, pp. 287–296, Apr. 2010.
 - [265] G. Umpierrez, S. Tofé Povedano, F. Pérez Manghi, L. Shurzinske, and V. Pechtner, "Efficacy and Safety of Dulaglutide Monotherapy Versus Metformin in Type 2 Diabetes in a Randomized Controlled Trial (AWARD-3)," *Diabetes Care*, vol. 37, no. 8, pp. 2168–2176, Aug. 2014.
 - [266] M. A. Nauck *et al.*, "Efficacy and safety of once-weekly GLP-1 receptor agonist albiglutide (HARMONY 2): 52 week primary endpoint results from a randomised, placebo-controlled trial in patients with type 2 diabetes mellitus inadequately controlled with diet and exercise," *Diabetologia*, vol. 59, no. 2, pp. 266–274, Feb. 2016.
 - [267] K. M. Dungan *et al.*, "Once-weekly dulaglutide versus once-daily liraglutide in metformin-treated patients with type 2 diabetes (AWARD-6): a randomised, open-label, phase 3, non-inferiority trial," *Lancet*, vol. 384, no. 9951, pp. 1349–1357, Oct. 2014.
 - [268] R. E. Pratley *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.*, vol. 2, no. 4, pp. 289–97, Apr. 2014.
 - [269] C. Chaudhury *et al.*, "The Major Histocompatibility Complex-related Fc Receptor for IgG (FcRn) Binds Albumin and Prolongs Its Lifespan," *J. Exp. Med.*, vol. 197, no. 3, pp. 315–322, Feb. 2003.
 - [270] T. Suzuki *et al.*, "Importance of Neonatal FcR in Regulating the Serum Half-Life of Therapeutic Proteins Containing the Fc Domain of Human IgG1: A Comparative Study of the Affinity of Monoclonal Antibodies and Fc-Fusion Proteins to Human Neonatal FcR," *J. Immunol.*, vol. 184, no. 4, pp. 1968–1976, Feb. 2010.
 - [271] J. M. Trujillo and W. Nuffer, "GLP-1 Receptor Agonists for Type 2 Diabetes Mellitus: Recent Developments and Emerging Agents," *Pharmacother. J. Hum. Pharmacol. Drug Ther.*, vol. 34, no. 11, pp. 1174–1186, Nov. 2014.
 - [272] R. Gentilella, C. Bianchi, A. Rossi, and C. M. Rotella, "Exenatide: a review from pharmacology to clinical practice," *Diabetes, Obes. Metab.*, vol. 11, no. 6, pp. 544–556, Jun. 2009.
 - [273] M. A. Nauck, G. Kemmeries, J. J. Holst, and J. J. Meier, "Rapid Tachyphylaxis of the Glucagon-Like Peptide 1-Induced Deceleration of Gastric Emptying in Humans," *Diabetes*, vol. 60, no. 5, pp. 1561–1565, May 2011.
 - [274] P. S. Denker and P. E. Dimarco, "Exenatide (Exendin-4)-Induced Pancreatitis: A case report," *Diabetes Care*, vol. 29, no. 2, pp. 471–471, Feb. 2006.
 - [275] W. Ayoub, A. Kumar, H. Naguib, and H. Taylor, "Exenatide-Induced Acute Pancreatitis," *Endocr. Pract.*, vol. 16, no. 1, pp. 80–83, Jan. 2010.
 - [276] B. Gier, A. V. Matveyenko, D. Kirakossian, D. Dawson, S. M. Dry, and P. C. Butler, "Chronic GLP-1 Receptor Activation by Exendin-4 Induces Expansion of Pancreatic Duct Glands in Rats and Accelerates Formation of Dysplastic Lesions and Chronic Pancreatitis in the Kras G12D Mouse Model," *Diabetes*, vol. 61, no. 5, pp. 1250–1262, May 2012.
 - [277] J. S. Nachnani *et al.*, "Biochemical and histological effects of exendin-4 (exenatide) on the rat pancreas," *Diabetologia*, vol. 53, no. 1, pp. 153–159, Jan. 2010.
 - [278] J. A. Koehler, L. L. Baggio, B. J. Lamont, S. Ali, and D. J. Drucker, "Glucagon-Like Peptide-1 Receptor Activation Modulates Pancreatitis-Associated Gene Expression But Does Not Modify the Susceptibility to Experimental Pancreatitis in Mice," *Diabetes*, vol. 58, no. 9, pp. 2148–2161, Sep. 2009.
 - [279] K. Tatarkiewicz *et al.*, "Exenatide does not evoke pancreatitis and attenuates chemically induced pancreatitis in normal and diabetic rodents," *Am. J. Physiol. Metab.*, vol. 299, no. 6, pp. E1076–E1086, Dec. 2010.
 - [280] N. C. B. Nyborg, A.-M. Mølck, L. W. Madsen, and L. Bjerre Knudsen, "The Human GLP-1 Analog Liraglutide and the Pancreas," *Diabetes*, vol. 61, no. 5, pp. 1243–1249, May 2012.
 - [281] M. Elashoff, A. V. Matveyenko, B. Gier, R. Elashoff, and P. C. Butler, "Pancreatitis, Pancreatic, and Thyroid Cancer With Glucagon-Like Peptide-1-Based Therapies," *Gastroenterology*, vol.

- 141, no. 1, pp. 150–156, Jul. 2011.
- [282] S. Singh, H.-Y. Chang, T. M. Richards, J. P. Weiner, J. M. Clark, and J. B. Segal, "Glucagonlike Peptide 1–Based Therapies and Risk of Hospitalization for Acute Pancreatitis in Type 2 Diabetes Mellitus," *JAMA Intern. Med.*, vol. 173, no. 7, p. 534, Apr. 2013.
 - [283] D. Soranna *et al.*, "Incretin-based drugs and risk of acute pancreatitis: A nested-case control study within a healthcare database," *Diabetes Res. Clin. Pract.*, vol. 108, no. 2, pp. 243–249, May 2015.
 - [284] L. Li *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*, vol. 348, no. apr15 2, pp. g2366–g2366, Apr. 2014.
 - [285] H. Storgaard, F. Cold, L. L. Gluud, T. Vilsbøll, and F. K. Knop, "Glucagon-like peptide-1 receptor agonists and risk of acute pancreatitis in patients with type 2 diabetes," *Diabetes, Obes. Metab.*, vol. 19, no. 6, pp. 906–908, Jun. 2017.
 - [286] A. G. Egan *et al.*, "Pancreatic Safety of Incretin-Based Drugs — FDA and EMA Assessment," *N. Engl. J. Med.*, vol. 370, no. 9, pp. 794–797, Feb. 2014.
 - [287] L. Bjerre Knudsen *et al.*, "Glucagon-Like Peptide-1 Receptor Agonists Activate Rodent Thyroid C-Cells Causing Calcitonin Release and C-Cell Proliferation," *Endocrinology*, vol. 151, no. 4, pp. 1473–1486, Apr. 2010.
 - [288] L. Hegedüs, A. C. Moses, M. Zdravkovic, T. Le Thi, and G. H. Daniels, "GLP-1 and Calcitonin Concentration in Humans: Lack of Evidence of Calcitonin Release from Sequential Screening in over 5000 Subjects with Type 2 Diabetes or Nondiabetic Obese Subjects Treated with the Human GLP-1 Analog, Liraglutide," *J. Clin. Endocrinol. Metab.*, vol. 96, no. 3, pp. 853–860, Mar. 2011.
 - [289] B. Thorens, "Expression cloning of the pancreatic beta cell receptor for the gluco-incretin hormone glucagon-like peptide 1," *Proc. Natl. Acad. Sci.*, vol. 89, no. 18, pp. 8641–8645, Sep. 1992.
 - [290] C. de Graaf *et al.*, "Extending the Structural View of Class B GPCRs," *Trends Biochem. Sci.*, vol. 42, no. 12, pp. 946–960, Dec. 2017.
 - [291] L.-H. Zhao *et al.*, "Differential Requirement of the Extracellular Domain in Activation of Class B G Protein-coupled Receptors," *J. Biol. Chem.*, vol. 291, no. 29, pp. 15119–15130, Jul. 2016.
 - [292] D. Hilger, M. Masureel, and B. K. Kobilka, "Structure and dynamics of GPCR signaling complexes," *Nat. Struct. Mol. Biol.*, vol. 25, no. 1, pp. 4–12, Jan. 2018.
 - [293] S. M. Khan *et al.*, "The Expanding Roles of G Subunits in G Protein-Coupled Receptor Signaling and Drug Action," *Pharmacol. Rev.*, vol. 65, no. 2, pp. 545–577, Apr. 2013.
 - [294] M. B. Wheeler, M. Lu, J. S. Dillon, X. H. Leng, C. Chen, and A. E. Boyd, "Functional expression of the rat glucagon-like peptide-I receptor, evidence for coupling to both adenylyl cyclase and phospholipase-C," *Endocrinology*, vol. 133, no. 1, pp. 57–62, Jul. 1993.
 - [295] C. Widmann, E. Bürki, W. Dolci, and B. Thorens, "Signal transduction by the cloned glucagon-like peptide-1 receptor: comparison with signaling by the endogenous receptors of beta cell lines," *Mol. Pharmacol.*, vol. 45, no. 5, pp. 1029–35, May 1994.
 - [296] C. Montrose-Rafizadeh *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*, vol. 140, no. 3, pp. 1132–1140, Mar. 1999.
 - [297] L. B. Lester, L. K. Langeberg, and J. D. Scott, "Anchoring of protein kinase A facilitates hormone-mediated insulin secretion," *Proc. Natl. Acad. Sci.*, vol. 94, no. 26, pp. 14942–14947, Dec. 1997.
 - [298] G. G. Holz, "Epac: A new cAMP-binding protein in support of glucagon-like peptide-1 receptor-mediated signal transduction in the pancreatic beta-cell," *Diabetes*, vol. 53, no. 1, pp. 5–13, Jan. 2004.
 - [299] P. E. Light, J. E. Manning Fox, M. J. Riedel, and M. B. Wheeler, "Glucagon-Like Peptide-1 Inhibits Pancreatic ATP-Sensitive Potassium Channels via a Protein Kinase A- and ADP-Dependent Mechanism," *Mol. Endocrinol.*, vol. 16, no. 9, pp. 2135–2144, Sep. 2002.
 - [300] G. Kang, C. A. Leech, O. G. Chepurny, W. A. Coetzee, and G. G. Holz, "Role of the cAMP sensor Epac as a determinant of K ATP channel ATP sensitivity in human pancreatic β -cells and rat INS-1 cells," *J. Physiol.*, vol. 586, no. 5, pp. 1307–1319, Mar. 2008.
 - [301] P. E. MacDonald and M. B. Wheeler, "Voltage-dependent K⁺ channels in pancreatic beta cells: Role, regulation and potential as therapeutic targets," *Diabetologia*, vol. 46, no. 8, pp. 1046–1062, Aug. 2003.
 - [302] G. Kang *et al.*, "A cAMP and Ca²⁺ coincidence detector in support of Ca²⁺-induced Ca²⁺ release in mouse pancreatic β cells," *J. Physiol.*, vol. 566, no. 1, pp. 173–188, Jul. 2005.

- [303] T. TSUBOI, G. da SILVA XAVIER, G. G. HOLZ, L. S. JOUAVILLE, A. P. THOMAS, and G. A. RUTTER, "Glucagon-like peptide-1 mobilizes intracellular Ca²⁺ and stimulates mitochondrial ATP synthesis in pancreatic MIN6 beta-cells," *Biochem. J.*, vol. 369, no. 2, pp. 287–299, Jan. 2003.
- [304] M. Shigeto *et al.*, "GLP-1 stimulates insulin secretion by PKC-dependent TRPM4 and TRPM5 activation," *J. Clin. Invest.*, vol. 125, no. 12, pp. 4714–4728, Nov. 2015.
- [305] J. Liu and Z. P. Pang, "Glucagon-like peptide-1 drives energy metabolism on the synaptic highway," *FEBS J.*, vol. 283, no. 24, pp. 4413–4423, Dec. 2016.
- [306] M. R. Hayes *et al.*, "Intracellular Signals Mediating the Food Intake-Suppressive Effects of Hindbrain Glucagon-like Peptide-1 Receptor Activation," *Cell Metab.*, vol. 13, no. 3, pp. 320–330, Mar. 2011.
- [307] J. Liu *et al.*, "Enhanced AMPA Receptor Trafficking Mediates the Anorexigenic Effect of Endogenous Glucagon-like Peptide-1 in the Paraventricular Hypothalamus," *Neuron*, vol. 96, no. 4, pp. 897–909.e5, Nov. 2017.
- [308] X.-F. Wang, J.-J. Liu, J. Xia, J. Liu, V. Mirabella, and Z. P. Pang, "Endogenous Glucagon-like Peptide-1 Suppresses High-Fat Food Intake by Reducing Synaptic Drive onto Mesolimbic Dopamine Neurons," *Cell Rep.*, vol. 12, no. 5, pp. 726–733, Aug. 2015.
- [309] E. G. Miettlicki-Baase *et al.*, "The food intake-suppressive effects of glucagon-like peptide-1 receptor signaling in the ventral tegmental area are mediated by AMPA/kainate receptors," *Am. J. Physiol. Metab.*, vol. 305, no. 11, pp. E1367–E1374, Dec. 2013.
- [310] E. G. Miettlicki-Baase *et al.*, "Glucagon-Like Peptide-1 Receptor Activation in the Nucleus Accumbens Core Suppresses Feeding by Increasing Glutamatergic AMPA/Kainate Signaling," *J. Neurosci.*, vol. 34, no. 20, pp. 6985–6992, May 2014.
- [311] O. Babateen, S. V. Korol, Z. Jin, A. K. Bhandage, A. Ahemaiti, and B. Birnir, "Liraglutide modulates GABAergic signaling in rat hippocampal CA3 pyramidal neurons predominantly by presynaptic mechanism," *BMC Pharmacol. Toxicol.*, vol. 18, no. 1, p. 83, Dec. 2017.
- [312] S. V. Korol, Z. Jin, O. Babateen, and B. Birnir, "GLP-1 and Exendin-4 Transiently Enhance GABA A Receptor-Mediated Synaptic and Tonic Currents in Rat Hippocampal CA3 Pyramidal Neurons," *Diabetes*, vol. 64, no. 1, pp. 79–89, Jan. 2015.
- [313] C. Acuna-Goycolea, "Glucagon-Like Peptide 1 Excites Hypocretin/Orexin Neurons by Direct and Indirect Mechanisms: Implications for Viscera-Mediated Arousal," *J. Neurosci.*, vol. 24, no. 37, pp. 8141–8152, Sep. 2004.
- [314] J. S. Smith and S. Rajagopal, "The β -Arrestins: Multifunctional Regulators of G Protein-coupled Receptors," *J. Biol. Chem.*, vol. 291, no. 17, pp. 8969–8977, Apr. 2016.
- [315] J. L. Benovic, H. Kuhn, I. Weyand, J. Codina, M. G. Caron, and R. J. Lefkowitz, "Functional desensitization of the isolated beta-adrenergic receptor by the beta-adrenergic receptor kinase: potential role of an analog of the retinal protein arrestin (48-kDa protein).," *Proc. Natl. Acad. Sci.*, vol. 84, no. 24, pp. 8879–8882, Dec. 1987.
- [316] O. B. Goodman *et al.*, " β -Arrestin acts as a clathrin adaptor in endocytosis of the β 2-adrenergic receptor," *Nature*, vol. 383, no. 6599, pp. 447–450, Oct. 1996.
- [317] S. A. Laporte, R. H. Oakley, J. A. Holt, L. S. Barak, and M. G. Caron, "The Interaction of β -Arrestin with the AP-2 Adaptor Is Required for the Clustering of β 2 -Adrenergic Receptor into Clathrin-coated Pits," *J. Biol. Chem.*, vol. 275, no. 30, pp. 23120–23126, Jul. 2000.
- [318] L. M. Luttrell *et al.*, "Manifold roles of β -arrestins in GPCR signaling elucidated with siRNA and CRISPR/Cas9," *Sci. Signal.*, vol. 11, no. 549, p. eaat7650, Sep. 2018.
- [319] M. Grundmann *et al.*, "Lack of beta-arrestin signaling in the absence of active G proteins.," *Nat. Commun.*, vol. 9, no. 1, p. 341, 2018.
- [320] M. O'Hayre *et al.*, "Genetic evidence that β -arrestins are dispensable for the initiation of β 2 -adrenergic receptor signaling to ERK," *Sci. Signal.*, vol. 10, no. 484, p. eaal3395, Jun. 2017.
- [321] R. Jorgensen, L. Martini, T. W. Schwartz, and C. E. Elling, "Characterization of Glucagon-Like Peptide-1 Receptor β -Arrestin 2 Interaction: A High-Affinity Receptor Phenotype," *Mol. Endocrinol.*, vol. 19, no. 3, pp. 812–823, Mar. 2005.
- [322] N. Sonoda, T. Imamura, T. Yoshizaki, J. L. Babendure, J.-C. Lu, and J. M. Olefsky, "Beta-Arrestin-1 mediates glucagon-like peptide-1 signaling to insulin secretion in cultured pancreatic beta cells.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 105, no. 18, pp. 6614–9, May 2008.
- [323] B. Jones *et al.*, "Targeting GLP-1 receptor trafficking to improve agonist efficacy.," *Nat. Commun.*, vol. 9, no. 1, p. 1602, 2018.
- [324] J. Quoyer *et al.*, "GLP-1 Mediates Antiapoptotic Effect by Phosphorylating Bad through a β -Arrestin 1-mediated ERK1/2 Activation in Pancreatic β -Cells," *J. Biol. Chem.*, vol. 285, no. 3, pp. 1989–2002, Jan. 2010.

- [325] H. C. FEHMANN and J. F. HABENER, "Homologous Desensitization of the Insulinotropic Glucagon-Like Peptide-I(7–37) Receptor on Insulinoma (HIT-T15) Cells*," *Endocrinology*, vol. 128, no. 6, pp. 2880–2888, Jun. 1991.
- [326] C. Widmann, W. Dolci, and B. Thorens, "Desensitization and phosphorylation of the glucagon-like peptide-1 (GLP-1) receptor by GLP-1 and 4-phorbol 12-myristate 13-acetate.," *Mol. Endocrinol.*, vol. 10, no. 1, pp. 62–75, Jan. 1996.
- [327] S. N. Roed *et al.*, "Real-time trafficking and signaling of the glucagon-like peptide-1 receptor," *Mol. Cell. Endocrinol.*, vol. 382, no. 2, pp. 938–949, Feb. 2014.
- [328] A. Thompson and V. Kanamarlapudi, "Agonist-induced internalisation of the glucagon-like peptide-1 receptor is mediated by the Gαq pathway," *Biochem. Pharmacol.*, vol. 93, no. 1, pp. 72–84, Jan. 2015.
- [329] C. A. Syme, L. Zhang, and A. Bisello, "Caveolin-1 Regulates Cellular Trafficking and Function of the Glucagon-Like Peptide 1 Receptor," *Mol. Endocrinol.*, vol. 20, no. 12, pp. 3400–3411, Dec. 2006.
- [330] S. Ferrandon *et al.*, "Sustained cyclic AMP production by parathyroid hormone receptor endocytosis," *Nat. Chem. Biol.*, vol. 5, no. 10, pp. 734–742, Oct. 2009.
- [331] L. A. Merriam, C. N. Baran, B. M. Girard, J. C. Hardwick, V. May, and R. L. Parsons, "Pituitary Adenylate Cyclase 1 Receptor Internalization and Endosomal Signaling Mediate the Pituitary Adenylate Cyclase Activating Polypeptide-Induced Increase in Guinea Pig Cardiac Neuron Excitability," *J. Neurosci.*, vol. 33, no. 10, pp. 4614–4622, Mar. 2013.
- [332] T. N. Feinstein *et al.*, "Noncanonical Control of Vasopressin Receptor Type 2 Signaling by Retromer and Arrestin," *J. Biol. Chem.*, vol. 288, no. 39, pp. 27849–27860, Sep. 2013.
- [333] R. S. Kuna *et al.*, "Glucagon-like peptide-1 receptor-mediated endosomal cAMP generation promotes glucose-stimulated insulin secretion in pancreatic β-cells," *Am. J. Physiol. Metab.*, vol. 305, no. 2, pp. E161–E170, Jul. 2013.
- [334] S. N. Roed *et al.*, "Functional Consequences of Glucagon-like Peptide-1 Receptor Cross-talk and Trafficking," *J. Biol. Chem.*, vol. 290, no. 2, pp. 1233–1243, Jan. 2015.
- [335] P. Leff, "The two-state model of receptor activation.," *Trends Pharmacol. Sci.*, vol. 16, no. 3, pp. 89–97, Mar. 1995.
- [336] D. Wootten, A. Christopoulos, M. Marti-Solano, M. M. Babu, and P. M. Sexton, "Mechanisms of signalling and biased agonism in G protein-coupled receptors," *Nat. Rev. Mol. Cell Biol.*, vol. 19, no. 10, pp. 638–653, Oct. 2018.
- [337] J. D. Violin, A. L. Crombie, D. G. Soergel, and M. W. Lark, "Biased ligands at G-protein-coupled receptors: promise and progress," *Trends Pharmacol. Sci.*, vol. 35, no. 7, pp. 308–316, Jul. 2014.
- [338] J. J. Liu, R. Horst, V. Katritch, R. C. Stevens, and K. Wuthrich, "Biased Signaling Pathways in 2-Adrenergic Receptor Characterized by 19F-NMR," *Science (80-.)*, vol. 335, no. 6072, pp. 1106–1110, Mar. 2012.
- [339] J. Okude *et al.*, "Identification of a Conformational Equilibrium That Determines the Efficacy and Functional Selectivity of the μ-Opioid Receptor," *Angew. Chemie Int. Ed.*, vol. 54, no. 52, pp. 15771–15776, Dec. 2015.
- [340] J. Gundry, R. Glenn, P. Alagesan, and S. Rajagopal, "A Practical Guide to Approaching Biased Agonism at G Protein Coupled Receptors," *Front. Neurosci.*, vol. 11, Jan. 2017.
- [341] H. O. Onaran *et al.*, "Systematic errors in detecting biased agonism: Analysis of current methods and development of a new model-free approach.," *Sci. Rep.*, vol. 7, p. 44247, 2017.
- [342] M. C. Michel and S. J. Charlton, "Biased Agonism in Drug Discovery—Is It Too Soon to Choose a Path?," *Mol. Pharmacol.*, vol. 93, no. 4, pp. 259–265, Apr. 2018.
- [343] L. M. Bohn, R. J. Lefkowitz, R. R. Gainetdinov, K. Peppel, M. G. Caron, and F. T. Lin, "Enhanced morphine analgesia in mice lacking beta-arrestin 2.," *Science*, vol. 286, no. 5449, pp. 2495–8, Dec. 1999.
- [344] L. M. Bohn, R. R. Gainetdinov, F.-T. Lin, R. J. Lefkowitz, and M. G. Caron, "μ-Opioid receptor desensitization by β-arrestin-2 determines morphine tolerance but not dependence," *Nature*, vol. 408, no. 6813, pp. 720–723, Dec. 2000.
- [345] Y. Li *et al.*, "Improvement of Morphine-Mediated Analgesia by Inhibition of β-Arrestin 2 Expression in Mice Periaqueductal Gray Matter," *Int. J. Mol. Sci.*, vol. 10, no. 3, pp. 954–963, Mar. 2009.
- [346] C.-H. Yang, H.-W. Huang, K.-H. Chen, Y.-S. Chen, S.-M. Sheen-Chen, and C.-R. Lin, "Antinociceptive potentiation and attenuation of tolerance by intrathecal β-arrestin 2 small interfering RNA in rats," *Br. J. Anaesth.*, vol. 107, no. 5, pp. 774–781, Nov. 2011.
- [347] S. M. DeWire *et al.*, "A G Protein-Biased Ligand at the μ-Opioid Receptor Is Potently Analgesic

- with Reduced Gastrointestinal and Respiratory Dysfunction Compared with Morphine," *J. Pharmacol. Exp. Ther.*, vol. 344, no. 3, pp. 708–717, Mar. 2013.
- [348] N. Singla *et al.*, "A randomized, Phase IIb study investigating oliceridine (TRV130), a novel μ -receptor G-protein pathway selective (μ -GPS) modulator, for the management of moderate to severe acute pain following abdominoplasty," *J. Pain Res.*, vol. Volume 10, pp. 2413–2424, Oct. 2017.
- [349] E. R. Viscusi, F. Skobieranda, D. G. Soergel, E. Cook, D. A. Burt, and N. Singla, "APOLLO-1: a randomized placebo and active-controlled phase III study investigating oliceridine (TRV130), a G protein-biased ligand at the μ -opioid receptor, for management of moderate-to-severe acute pain following bunionectomy," *J. Pain Res.*, vol. Volume 12, pp. 927–943, Mar. 2019.
- [350] A. Manglik *et al.*, "Structure-based discovery of opioid analgesics with reduced side effects," *Nature*, vol. 537, no. 7619, pp. 185–190, Sep. 2016.
- [351] S. M. Park *et al.*, "Effects of β -Arrestin-Biased Dopamine D2 Receptor Ligands on Schizophrenia-Like Behavior in Hypoglutamatergic Mice," *Neuropsychopharmacology*, vol. 41, no. 3, pp. 704–715, Feb. 2016.
- [352] R. W. Walters *et al.*, " β -Arrestin1 mediates nicotinic acid-induced flushing, but not its antilipolytic effect, in mice," *J. Clin. Invest.*, vol. 119, no. 5, pp. 1312–1321, May 2009.
- [353] D. Gesty-Palmer *et al.*, "A β -Arrestin-Biased Agonist of the Parathyroid Hormone Receptor (PTH1R) Promotes Bone Formation Independent of G Protein Activation," *Sci. Transl. Med.*, vol. 1, no. 1, pp. 1ra1–1ra1, Oct. 2009.
- [354] G. Becker, R. Bolbos, N. Costes, J. Redouté, A. Newman-Tancredi, and L. Zimmer, "Selective serotonin 5-HT_{1A} receptor biased agonists elicit distinct brain activation patterns: a pharmacMRI study," *Sci. Rep.*, vol. 6, no. 1, p. 26633, Jul. 2016.
- [355] M. Jastrzębska-Więsek *et al.*, "Activity of Serotonin 5-HT_{1A} Receptor Biased Agonists in Rat: Anxiolytic and Antidepressant-like properties," *ACS Chem. Neurosci.*, vol. 9, no. 5, pp. 1040–1050, May 2018.
- [356] C. L. Schmid, K. M. Raehal, and L. M. Bohn, "Agonist-directed signaling of the serotonin 2A receptor depends on β -arrestin-2 interactions in vivo," *Proc. Natl. Acad. Sci.*, vol. 105, no. 3, pp. 1079–1084, Jan. 2008.
- [357] E. A. Vecchio, J.-A. Baltos, A. T. N. Nguyen, A. Christopoulos, P. J. White, and L. T. May, "New paradigms in adenosine receptor pharmacology: allosterity, oligomerization and biased agonism," *Br. J. Pharmacol.*, vol. 175, no. 21, pp. 4036–4046, Nov. 2018.
- [358] J. W. Wisler *et al.*, "A unique mechanism of beta-blocker action: Carvedilol stimulates β -arrestin signaling," *Proc. Natl. Acad. Sci.*, vol. 104, no. 42, pp. 16657–16662, Oct. 2007.
- [359] R. Carr *et al.*, " β -arrestin-biased signaling through the β_2 -adrenergic receptor promotes cardiomyocyte contraction," *Proc. Natl. Acad. Sci.*, vol. 113, no. 28, pp. E4107–E4116, Jul. 2016.
- [360] J. Wang *et al.*, "Gai is required for carvedilol-induced β_1 adrenergic receptor β -arrestin biased signaling," *Nat. Commun.*, vol. 8, no. 1, p. 1706, Dec. 2017.
- [361] J. D. Violin *et al.*, "Selectively Engaging β -Arrestins at the Angiotensin II Type 1 Receptor Reduces Blood Pressure and Increases Cardiac Performance," *J. Pharmacol. Exp. Ther.*, vol. 335, no. 3, pp. 572–579, Dec. 2010.
- [362] G. Boerrigter, M. W. Lark, E. J. Whalen, D. G. Soergel, J. D. Violin, and J. C. Burnett, "Cardiorenal Actions of TRV120027, a Novel β -Arrestin-Biased Ligand at the Angiotensin II Type I Receptor, in Healthy and Heart Failure Canines," *Circ. Hear. Fail.*, vol. 4, no. 6, pp. 770–778, Nov. 2011.
- [363] P. S. Pang *et al.*, "Biased ligand of the angiotensin II type 1 receptor in patients with acute heart failure: a randomized, double-blind, placebo-controlled, phase IIB, dose ranging trial (BLAST-AHF)," *Eur. Heart J.*, vol. 38, no. 30, pp. 2364–2373, Aug. 2017.
- [364] O.-E. Brodde, " β -adrenoceptor blocker treatment and the cardiac β -adrenoceptor-G-protein(s)-adenylyl cyclase system in chronic heart failure," *Naunyn. Schmiedeberg's Arch. Pharmacol.*, vol. 374, no. 5–6, pp. 361–372, Feb. 2007.
- [365] C. Koole *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.*, vol. 78, no. 3, pp. 456–465, Sep. 2010.
- [366] D. Wootten *et al.*, "The Extracellular Surface of the GLP-1 Receptor Is a Molecular Trigger for Biased Agonism," *Cell*, vol. 165, no. 7, pp. 1632–1643, Jun. 2016.
- [367] M. M. Fletcher *et al.*, "Glucagon-like peptide-1 receptor internalisation controls spatiotemporal signalling mediated by biased agonists," *Biochem. Pharmacol.*, vol. 156, pp. 406–419, Oct.

- 2018.
- [368] D. Wootten *et al.*, "Differential Activation and Modulation of the Glucagon-Like Peptide-1 Receptor by Small Molecule Ligands," *Mol. Pharmacol.*, vol. 83, no. 4, pp. 822–834, Apr. 2013.
 - [369] M. V Hager, L. Clydesdale, S. H. Gellman, P. M. Sexton, and D. Wootten, "Characterization of signal bias at the GLP-1 receptor induced by backbone modification of GLP-1," *Biochem. Pharmacol.*, vol. 136, pp. 99–108, 2017.
 - [370] H. Zhang *et al.*, "Autocrine selection of a GLP-1R G-protein biased agonist with potent antidiabetic effects.," *Nat. Commun.*, vol. 6, p. 8918, Dec. 2015.
 - [371] D. Wootten, J. Simms, L. J. Miller, A. Christopoulos, and P. M. Sexton, "Polar transmembrane interactions drive formation of ligand-specific and signal pathway-biased family B G protein-coupled receptor conformations," *Proc. Natl. Acad. Sci.*, vol. 110, no. 13, pp. 5211–5216, Mar. 2013.
 - [372] D. Wootten *et al.*, "Key interactions by conserved polar amino acids located at the transmembrane helical boundaries in Class B GPCRs modulate activation, effector specificity and biased signalling in the glucagon-like peptide-1 receptor," *Biochem. Pharmacol.*, vol. 118, pp. 68–87, Oct. 2016.
 - [373] D. Wootten *et al.*, "A Hydrogen-Bonded Polar Network in the Core of the Glucagon-Like Peptide-1 Receptor Is a Fulcrum for Biased Agonism: Lessons from Class B Crystal Structures," *Mol. Pharmacol.*, vol. 89, no. 3, pp. 335–347, Jan. 2016.
 - [374] S. Lei *et al.*, "Two distinct domains of the glucagon-like peptide-1 receptor control peptide-mediated biased agonism," *J. Biol. Chem.*, vol. 293, no. 24, pp. 9370–9387, Jun. 2018.
 - [375] Y.-L. Liang *et al.*, "Phase-plate cryo-EM structure of a biased agonist-bound human GLP-1 receptor-Gs complex.," *Nature*, vol. 555, no. 7694, pp. 121–125, 2018.
 - [376] A. Jazayeri *et al.*, "Crystal structure of the GLP-1 receptor bound to a peptide agonist.," *Nature*, vol. 546, no. 7657, pp. 254–258, 2017.
 - [377] Y. Zhang *et al.*, "Cryo-EM structure of the activated GLP-1 receptor in complex with a G protein.," *Nature*, vol. 546, no. 7657, pp. 248–253, 2017.
 - [378] D. M. Freed *et al.*, "EGFR Ligands Differentially Stabilize Receptor Dimers to Specify Signaling Kinetics," *Cell*, vol. 171, no. 3, pp. 683–695.e18, Oct. 2017.
 - [379] A. Nazarians-Armavil, J. A. Chalmers, C. B. Lee, W. Ye, and D. D. Belsham, "Cellular insulin resistance disrupts hypothalamic mHypoA-POMC/GFP neuronal signaling pathways.," *J. Endocrinol.*, vol. 220, no. 1, pp. 13–24, Jan. 2014.
 - [380] T. Kenakin, C. Watson, V. Muniz-Medina, A. Christopoulos, and S. Novick, "A simple method for quantifying functional selectivity and agonist bias.," *ACS Chem. Neurosci.*, vol. 3, no. 3, pp. 193–203, Mar. 2012.
 - [381] J. Ast *et al.*, "LUXendins reveal endogenous glucagon-like peptide-1 receptor distribution and dynamics," 2019.
 - [382] A. Ertürk *et al.*, "Three-dimensional imaging of solvent-cleared organs using 3DISCO.," *Nat. Protoc.*, vol. 7, no. 11, pp. 1983–95, Nov. 2012.
 - [383] K. Franklin and G. Paxinos, *The Mouse Brain in Stereotaxic Coordinates*. .
 - [384] J. P. H. Wilding and K. Hardy, "Glucagon-like peptide-1 analogues for type 2 diabetes.," *BMJ*, vol. 342, p. d410, Feb. 2011.
 - [385] J. E. Ayala *et al.*, "Standard operating procedures for describing and performing metabolic tests of glucose homeostasis in mice.," *Dis. Model. Mech.*, vol. 3, no. 9–10, pp. 525–34.
 - [386] R. Mentlein, B. Gallwitz, and W. E. Schmidt, "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.*, vol. 214, no. 3, pp. 829–35, Jun. 1993.
 - [387] U. Ritzel *et al.*, "A synthetic glucagon-like peptide-1 analog with improved plasma stability.," *J. Endocrinol.*, vol. 159, no. 1, pp. 93–102, Oct. 1998.
 - [388] C. F. Deacon, L. B. Knudsen, K. Madsen, F. C. Wiberg, O. Jacobsen, and J. J. Holst, "Dipeptidyl peptidase IV resistant analogues of glucagon-like peptide-1 which have extended metabolic stability and improved biological activity," *Diabetologia*, vol. 41, no. 3, pp. 271–278, Feb. 1998.
 - [389] C. L. Schmid *et al.*, "Bias Factor and Therapeutic Window Correlate to Predict Safer Opioid Analgesics.," *Cell*, vol. 171, no. 5, pp. 1165–1175.e13, Nov. 2017.
 - [390] Z. Zhao, A. R. Nelson, C. Betsholtz, and B. V. Zlokovic, "Establishment and Dysfunction of the Blood-Brain Barrier," *Cell*, vol. 163, no. 5, pp. 1064–1078, Nov. 2015.
 - [391] E. Balland *et al.*, "Hypothalamic tanycytes are an ERK-gated conduit for leptin into the brain.," *Cell Metab.*, vol. 19, no. 2, pp. 293–301, Feb. 2014.

- [392] M. Uhlen *et al.*, "Tissue-based map of the human proteome," *Science* (80-.), vol. 347, no. 6220, pp. 1260419–1260419, Jan. 2015.
- [393] K. E. Warren, "Beyond the Blood:Brain Barrier: The Importance of Central Nervous System (CNS) Pharmacokinetics for the Treatment of CNS Tumors, Including Diffuse Intrinsic Pontine Glioma," *Front. Oncol.*, vol. 8, Jul. 2018.
- [394] R. Daneman and A. Prat, "The blood-brain barrier.," *Cold Spring Harb. Perspect. Biol.*, vol. 7, no. 1, p. a020412, Jan. 2015.
- [395] N. J. Abbott, A. A. K. Patabendige, D. E. M. Dolman, S. R. Yusof, and D. J. Begley, "Structure and function of the blood–brain barrier," *Neurobiol. Dis.*, vol. 37, no. 1, pp. 13–25, Jan. 2010.
- [396] E. M. Rodríguez, J. L. Blázquez, and M. Guerra, "The design of barriers in the hypothalamus allows the median eminence and the arcuate nucleus to enjoy private milieus: The former opens to the portal blood and the latter to the cerebrospinal fluid," *Peptides*, vol. 31, no. 4, pp. 757–776, Apr. 2010.
- [397] B. Engelhardt and L. Sorokin, "The blood-brain and the blood-cerebrospinal fluid barriers: function and dysfunction.," *Semin. Immunopathol.*, vol. 31, no. 4, pp. 497–511, Nov. 2009.
- [398] F. Langlet, A. Mullier, S. G. Bouret, V. Prevot, and B. Dehouck, "Tanycyte-like cells form a blood-cerebrospinal fluid barrier in the circumventricular organs of the mouse brain.," *J. Comp. Neurol.*, vol. 521, no. 15, pp. 3389–405, Oct. 2013.
- [399] J. N. Campbell *et al.*, "A molecular census of arcuate hypothalamus and median eminence cell types.," *Nat. Neurosci.*, vol. 20, no. 3, pp. 484–496, Mar. 2017.
- [400] V. Prevot, B. Dehouck, A. Sharif, P. Ciofi, P. Giacobini, and J. Clasadonte, "The Versatile Tanycyte: A Hypothalamic Integrator of Reproduction and Energy Metabolism," *Endocr. Rev.*, vol. 39, no. 3, pp. 333–368, Jun. 2018.
- [401] S. Morita *et al.*, "Heterogeneous vascular permeability and alternative diffusion barrier in sensory circumventricular organs of adult mouse brain.," *Cell Tissue Res.*, vol. 363, no. 2, pp. 497–511, Feb. 2016.
- [402] A. Mullier, S. G. Bouret, V. Prevot, and B. Dehouck, "Differential distribution of tight junction proteins suggests a role for tanycytes in blood-hypothalamus barrier regulation in the adult mouse brain.," *J. Comp. Neurol.*, vol. 518, no. 7, pp. 943–62, Apr. 2010.
- [403] P. Ciofi *et al.*, "Brain-Endocrine Interactions: A Microvascular Route in the Mediobasal Hypothalamus," *Endocrinology*, vol. 150, no. 12, pp. 5509–5519, Dec. 2009.
- [404] P. Ciofi, "The arcuate nucleus as a circumventricular organ in the mouse," *Neurosci. Lett.*, vol. 487, no. 2, pp. 187–190, Jan. 2011.
- [405] F. Langlet *et al.*, "Tanycytic VEGF-A boosts blood-hypothalamus barrier plasticity and access of metabolic signals to the arcuate nucleus in response to fasting.," *Cell Metab.*, vol. 17, no. 4, pp. 607–17, Apr. 2013.
- [406] M. Schaeffer *et al.*, "Rapid sensing of circulating ghrelin by hypothalamic appetite-modifying neurons.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 110, no. 4, pp. 1512–7, Jan. 2013.
- [407] J. Praetorius and H. H. Damkier, "Transport across the choroid plexus epithelium.," *Am. J. Physiol. Cell Physiol.*, vol. 312, no. 6, pp. C673–C686, Jun. 2017.
- [408] R. Villaseñor, J. Lampe, M. Schwaninger, and L. Collin, "Intracellular transport and regulation of transcytosis across the blood-brain barrier.," *Cell. Mol. Life Sci.*, vol. 76, no. 6, pp. 1081–1092, Mar. 2019.
- [409] L. I. Goulatis and E. V Shusta, "Protein engineering approaches for regulating blood–brain barrier transcytosis," *Curr. Opin. Struct. Biol.*, vol. 45, pp. 109–115, Aug. 2017.
- [410] R. Villaseñor, M. Schilling, J. Sundaresan, Y. Lutz, and L. Collin, "Sorting Tubules Regulate Blood-Brain Barrier Transcytosis," *Cell Rep.*, vol. 21, no. 11, pp. 3256–3270, Dec. 2017.
- [411] V. M. Pulgar, "Transcytosis to Cross the Blood Brain Barrier, New Advancements and Challenges," *Front. Neurosci.*, vol. 12, Jan. 2019.
- [412] J. Niewoehner *et al.*, "Increased Brain Penetration and Potency of a Therapeutic Antibody Using a Monovalent Molecular Shuttle," *Neuron*, vol. 81, no. 1, pp. 49–60, Jan. 2014.
- [413] M. Yemisci, S. Caban, E. Fernandez-Megia, Y. Capan, P. Couvreur, and T. Dalkara, "Preparation and Characterization of Biocompatible Chitosan Nanoparticles for Targeted Brain Delivery of Peptides.," *Methods Mol. Biol.*, vol. 1727, pp. 443–454, 2018.
- [414] F. C. Thomas *et al.*, "Uptake of ANG1005, a novel paclitaxel derivative, through the blood-brain barrier into brain and experimental brain metastases of breast cancer.," *Pharm. Res.*, vol. 26, no. 11, pp. 2486–94, Nov. 2009.
- [415] N. Strazielle and J.-F. Gherzi-Egea, "Potential Pathways for CNS Drug Delivery Across the Blood-Cerebrospinal Fluid Barrier.," *Curr. Pharm. Des.*, vol. 22, no. 35, pp. 5463–5476, 2016.
- [416] E. Carro, "Choroid Plexus Megalin Is Involved in Neuroprotection by Serum Insulin-Like

- Growth Factor I," *J. Neurosci.*, vol. 25, no. 47, pp. 10884–10893, Nov. 2005.
- [417] M. O. Dietrich *et al.*, "Megalin mediates the transport of leptin across the blood-CSF barrier," *Neurobiol. Aging*, vol. 29, no. 6, pp. 902–912, Jun. 2008.
- [418] A. Cabral, S. Valdivia, G. Fernandez, M. Reynaldo, and M. Perello, "Divergent neuronal circuitries underlying acute orexigenic effects of peripheral or central ghrelin: critical role of brain accessibility," *J. Neuroendocrinol.*, vol. 26, no. 8, pp. 542–54, Aug. 2014.
- [419] G. Collden *et al.*, "Neonatal overnutrition causes early alterations in the central response to peripheral ghrelin," *Mol. Metab.*, vol. 4, no. 1, pp. 15–24, Jan. 2015.
- [420] H. Qosa, D. S. Miller, P. Pasinelli, and D. Trotti, "Regulation of ABC efflux transporters at blood-brain barrier in health and neurological disorders," *Brain Res.*, vol. 1628, no. Pt B, pp. 298–316, Dec. 2015.
- [421] W. A. Banks, "From blood-brain barrier to blood-brain interface: new opportunities for CNS drug delivery," *Nat. Rev. Drug Discov.*, vol. 15, no. 4, pp. 275–92, Apr. 2016.
- [422] J. M. R. Parepally, H. Mandula, and Q. R. Smith, "Brain Uptake of Nonsteroidal Anti-Inflammatory Drugs: Ibuprofen, Flurbiprofen, and Indomethacin," *Pharm. Res.*, vol. 23, no. 5, pp. 873–881, May 2006.
- [423] A. J. Kastin and V. Akerstrom, "Entry of exendin-4 into brain is rapid but may be limited at high doses," *Int. J. Obes. Relat. Metab. Disord.*, vol. 27, no. 3, pp. 313–8, Mar. 2003.
- [424] C. Hölscher, "Potential role of glucagon-like peptide-1 (GLP-1) in neuroprotection," *CNS Drugs*, vol. 26, no. 10, pp. 871–82, Oct. 2012.
- [425] S. Madsbad, "Review of head-to-head comparisons of glucagon-like peptide-1 receptor agonists," *Diabetes. Obes. Metab.*, vol. 18, no. 4, pp. 317–32, Apr. 2016.
- [426] K. M. Dungan *et al.*, "Once-weekly dulaglutide versus once-daily liraglutide in metformin-treated patients with type 2 diabetes (AWARD-6): a randomised, open-label, phase 3, non-inferiority trial," *Lancet (London, England)*, vol. 384, no. 9951, pp. 1349–57, Oct. 2014.
- [427] C. B. G. Salinas *et al.*, "Integrated Brain Atlas for Unbiased Mapping of Nervous System Effects Following Liraglutide Treatment," *Sci. Rep.*, vol. 8, no. 1, p. 10310, Jul. 2018.
- [428] M. Christensen *et al.*, "Transfer of liraglutide from blood to cerebrospinal fluid is minimal in patients with type 2 diabetes," *Int. J. Obes. (Lond.)*, vol. 39, no. 11, pp. 1651–4, Nov. 2015.
- [429] W. A. Banks, J. B. Jaspan, W. Huang, and A. J. Kastin, "Transport of insulin across the blood-brain barrier: saturability at euglycemic doses of insulin," *Peptides*, vol. 18, no. 9, pp. 1423–9, 1997.
- [430] W. A. Banks, A. J. Kastin, W. Huang, J. B. Jaspan, and L. M. Maness, "Leptin enters the brain by a saturable system independent of insulin," *Peptides*, vol. 17, no. 2, pp. 305–311, Jan. 1996.
- [431] T. T. Wager, X. Hou, P. R. Verhoest, and A. Villalobos, "Moving beyond rules: the development of a central nervous system multiparameter optimization (CNS MPO) approach to enable alignment of druglike properties," *ACS Chem. Neurosci.*, vol. 1, no. 6, pp. 435–49, Jun. 2010.
- [432] A. J. Kastin and V. Akerstrom, "Nonsaturable entry of neuropeptide Y into brain," *Am. J. Physiol.*, vol. 276, no. 3, pp. E479-82, 1999.
- [433] T. O. Price, W. K. Samson, M. L. Niehoff, and W. A. Banks, "Permeability of the blood-brain barrier to a novel satiety molecule nesfatin-1," *Peptides*, vol. 28, no. 12, pp. 2372–81, Dec. 2007.
- [434] H. Hsueh, W. Pan, and A. J. Kastin, "The fasting polypeptide FGF21 can enter brain from blood," *Peptides*, vol. 28, no. 12, pp. 2382–6, Dec. 2007.
- [435] A. J. Kastin and W. Pan, "Blood-brain barrier and feeding: regulatory roles of saturable transport systems for ingestive peptides," *Curr. Pharm. Des.*, vol. 14, no. 16, pp. 1615–9, 2008.
- [436] D. S. Richardson and J. W. Lichtman, "Clarifying Tissue Clearing," *Cell*, vol. 162, no. 2, pp. 246–257, Jul. 2015.
- [437] P. Ariel, "A beginner's guide to tissue clearing," *Int. J. Biochem. Cell Biol.*, vol. 84, pp. 35–39, Mar. 2017.
- [438] M. S. Viegas, T. C. Martins, F. Seco, and A. do Carmo, "An improved and cost-effective methodology for the reduction of autofluorescence in direct immunofluorescence studies on formalin-fixed paraffin-embedded tissues," *Eur. J. Histochem.*, vol. 51, no. 1, pp. 59–66.
- [439] L. Zhang and G. M. Thurber, "Quantitative Impact of Plasma Clearance and Down-regulation on GLP-1 Receptor Molecular Imaging," *Mol. Imaging Biol.*, vol. 18, no. 1, pp. 79–89, Feb. 2016.
- [440] L. Liang and D. Astruc, "The copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC) 'click'

- reaction and its applications. An overview," *Coord. Chem. Rev.*, vol. 255, no. 23–24, pp. 2933–2945, Dec. 2011.
- [441] S. M. Clardy *et al.*, "Rapid, high efficiency isolation of pancreatic β -cells.," *Sci. Rep.*, vol. 5, p. 13681, Sep. 2015.
 - [442] S. M. Clardy *et al.*, "Fluorescent exendin-4 derivatives for pancreatic β -cell analysis.," *Bioconjug. Chem.*, vol. 25, no. 1, pp. 171–7, Jan. 2014.
 - [443] T. Reiner *et al.*, "Accurate measurement of pancreatic islet beta-cell mass using a second-generation fluorescent exendin-4 analog.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 108, no. 31, pp. 12815–20, Aug. 2011.
 - [444] P.-Y. Yang *et al.*, "Engineering a long-acting, potent GLP-1 analog for microstructure-based transdermal delivery," *Proc. Natl. Acad. Sci.*, vol. 113, no. 15, pp. 4140–4145, Apr. 2016.
 - [445] S. Iwasaki *et al.*, "Mechanism-Based Pharmacokinetic/Pharmacodynamic Modeling of the Glucagon-Like Peptide-1 Receptor Agonist Exenatide to Characterize Its Antiobesity Effects in Diet-Induced Obese Mice," *J. Pharmacol. Exp. Ther.*, vol. 362, no. 3, pp. 441–449, Sep. 2017.
 - [446] S. A. Schnell, W. A. Staines, and M. W. Wessendorf, "Reduction of Lipofuscin-like Autofluorescence in Fluorescently Labeled Tissue," *J. Histochem. Cytochem.*, vol. 47, no. 6, pp. 719–730, Jun. 1999.
 - [447] N. Renier, Z. Wu, D. J. Simon, J. Yang, P. Ariel, and M. Tessier-Lavigne, "iDISCO: A Simple, Rapid Method to Immunolabel Large Tissue Samples for Volume Imaging," *Cell*, vol. 159, no. 4, pp. 896–910, Nov. 2014.
 - [448] K. Chung *et al.*, "Structural and molecular interrogation of intact biological systems.," *Nature*, vol. 497, no. 7449, pp. 332–7, May 2013.
 - [449] E. A. Susaki *et al.*, "Whole-brain imaging with single-cell resolution using chemical cocktails and computational analysis.," *Cell*, vol. 157, no. 3, pp. 726–39, Apr. 2014.
 - [450] W. Nishimura, A. Sakaue-Sawano, S. Takahashi, A. Miyawaki, K. Yasuda, and Y. Noda, "Optical clearing of the pancreas for visualization of mature β -cells and vessels in mice," *Islets*, vol. 10, no. 3, p. e1451282, May 2018.
 - [451] J. L. Fowler, S. S.-Y. Lee, Z. C. Wesner, S. K. Olehnik, S. J. Kron, and M. Hara, "Three-Dimensional Analysis of the Human Pancreas," *Endocrinology*, vol. 159, no. 3, pp. 1393–1400, Mar. 2018.
 - [452] J. Lovshin and D. Cherney, "GLP-1R Agonists and Endothelial Dysfunction: More Than Just Glucose Lowering?," *Diabetes*, vol. 64, no. 7, pp. 2319–2321, Jul. 2015.
 - [453] G. Pujadas and D. J. Drucker, "Vascular Biology of Glucagon Receptor Superfamily Peptides: Mechanistic and Clinical Relevance," *Endocr. Rev.*, vol. 37, no. 6, pp. 554–583, Dec. 2016.
 - [454] Y. Hou, S. A. Ernst, K. Heidenreich, and J. A. Williams, "Glucagon-like peptide-1 receptor is present in pancreatic acinar cells and regulates amylase secretion through cAMP.," *Am. J. Physiol. Gastrointest. Liver Physiol.*, vol. 310, no. 1, pp. G26–33, Jan. 2016.
 - [455] S. Chen *et al.*, "Post-treatment with PT302, a long-acting Exendin-4 sustained release formulation, reduces dopaminergic neurodegeneration in a 6-Hydroxydopamine rat model of Parkinson's disease.," *Sci. Rep.*, vol. 8, no. 1, p. 10722, Jul. 2018.
 - [456] N. Maolood and B. Meister, "Protein components of the blood–brain barrier (BBB) in the brainstem area postrema–nucleus tractus solitarius region," *J. Chem. Neuroanat.*, vol. 37, no. 3, pp. 182–195, May 2009.
 - [457] Q.-P. Wang, J.-L. Guan, W. Pan, A. J. Kastin, and S. Shioda, "A Diffusion Barrier Between the Area Postrema and Nucleus Tractus Solitarius," *Neurochem. Res.*, vol. 33, no. 10, pp. 2035–2043, Oct. 2008.
 - [458] W. A. Banks, "Peptides and the blood-brain barrier.," *Peptides*, vol. 72, pp. 16–9, Oct. 2015.
 - [459] H. F. Botfield *et al.*, "A glucagon-like peptide-1 receptor agonist reduces intracranial pressure in a rat model of hydrocephalus.," *Sci. Transl. Med.*, vol. 9, no. 404, Aug. 2017.
 - [460] S. Sonoda, C. Spee, E. Barron, S. J. Ryan, R. Kannan, and D. R. Hinton, "A protocol for the culture and differentiation of highly polarized human retinal pigment epithelial cells.," *Nat. Protoc.*, vol. 4, no. 5, pp. 662–73, 2009.
 - [461] J. M. Adams *et al.*, "Liraglutide Modulates Appetite and Body Weight Through Glucagon-Like Peptide 1 Receptor-Expressing Glutamatergic Neurons.," *Diabetes*, vol. 67, no. 8, pp. 1538–1548, 2018.
 - [462] J. Buggy and A. K. Jonhson, "Preoptic-hypothalamic periventricular lesions: thirst deficits and hypernatremia.," *Am. J. Physiol.*, vol. 233, no. 1, pp. R44–52, Jul. 1977.
 - [463] N. J. McKay, S. E. Kanoski, M. R. Hayes, and D. Daniels, "Glucagon-like peptide-1 receptor agonists suppress water intake independent of effects on food intake.," *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, vol. 301, no. 6, pp. R1755–64, Dec. 2011.

- [464] M. Kjaergaard, C. B. G. Salinas, J. F. Rehfeld, A. Secher, K. Raun, and B. S. Wulff, "PYY(3-36) and exendin-4 reduce food intake and activate neuronal circuits in a synergistic manner in mice," *Neuropeptides*, vol. 73, pp. 89–95, Feb. 2019.
- [465] Z. He *et al.*, "Direct and indirect effects of liraglutide on hypothalamic POMC and NPY/AgRP neurons – implications for energy balance and glucose control," *Mol. Metab.*, Jul. 2019.
- [466] M. A. Burmeister, J. Ayala, D. J. Drucker, and J. E. Ayala, "Central glucagon-like peptide 1 receptor-induced anorexia requires glucose metabolism-mediated suppression of AMPK and is impaired by central fructose," *Am. J. Physiol. Metab.*, vol. 304, no. 7, pp. E677–E685, Apr. 2013.
- [467] M. D. White, Z. W. Zhao, and N. Plachta, "In Vivo Imaging of Single Mammalian Cells in Development and Disease," *Trends Mol. Med.*, vol. 24, no. 3, pp. 278–293, Mar. 2018.
- [468] D. D. Belsham, F. Cai, H. Cui, S. R. Smukler, A. M. F. Salapatek, and L. Shkreta, "Generation of a phenotypic array of hypothalamic neuronal cell models to study complex neuroendocrine disorders," *Endocrinology*, vol. 145, no. 1, pp. 393–400, Jan. 2004.
- [469] P. S. Dalvi, A. Nazarians-Armavil, S. Tung, and D. D. Belsham, "Immortalized neurons for the study of hypothalamic function," *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, vol. 300, no. 5, pp. R1030–52, May 2011.
- [470] S. A. McFadden, J. A. Menchella, J. A. Chalmers, M.-L. Centeno, and D. D. Belsham, "Glucose responsiveness in a novel adult-derived GnRH cell line, mHypoA-GnRH/GFP: involvement of AMP-activated protein kinase," *Mol. Cell. Endocrinol.*, vol. 377, no. 1–2, pp. 65–74, Sep. 2013.
- [471] C. A. Aveleira *et al.*, "Neuropeptide Y stimulates autophagy in hypothalamic neurons," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 112, no. 13, pp. E1642–51, Mar. 2015.
- [472] A. K. Treen *et al.*, "Divergent Regulation of ER and Kiss Genes by 17 β -Estradiol in Hypothalamic ARC Versus AVPV Models," *Mol. Endocrinol.*, vol. 30, no. 2, pp. 217–33, Feb. 2016.
- [473] P. L. Mellon, J. J. Windle, P. C. Goldsmith, C. A. Padula, J. L. Roberts, and R. I. Weiner, "Immortalization of hypothalamic GnRH neurons by genetically targeted tumorigenesis," *Neuron*, vol. 5, no. 1, pp. 1–10, Jul. 1990.
- [474] H. Iwakura *et al.*, "Establishment of Leptin-Responsive Cell Lines from Adult Mouse Hypothalamus," *PLoS One*, vol. 11, no. 2, p. e0148639, Feb. 2016.
- [475] D. C. Jacobs, R. E. Veitch, and P. E. Chappell, "Evaluation of Immortalized AVPV- and Arcuate-Specific Neuronal Kisspeptin Cell Lines to Elucidate Potential Mechanisms of Estrogen Responsiveness and Temporal Gene Expression in Females," *Endocrinology*, vol. 157, no. 9, pp. 3410–9, 2016.
- [476] P. S. Dalvi, A. Nazarians-Armavil, M. J. Purser, and D. D. Belsham, "Glucagon-like peptide-1 receptor agonist, exendin-4, regulates feeding-associated neuropeptides in hypothalamic neurons in vivo and in vitro," *Endocrinology*, vol. 153, no. 5, pp. 2208–22, May 2012.
- [477] J. Gordon, S. Amini, and M. K. White, "General overview of neuronal cell culture," *Methods Mol. Biol.*, vol. 1078, pp. 1–8, 2013.
- [478] G. J. Brewer and J. R. Torricelli, "Isolation and culture of adult neurons and neurospheres," *Nat. Protoc.*, vol. 2, no. 6, pp. 1490–8, 2007.
- [479] N. Heeley *et al.*, "Rapid sensing of l-leucine by human and murine hypothalamic neurons: Neurochemical and mechanistic insights," *Mol. Metab.*, vol. 10, pp. 14–27, 2018.
- [480] A. A. Dunn-Meynell, V. H. Routh, L. Kang, L. Gaspers, and B. E. Levin, "Glucokinase Is the Likely Mediator of Glucosensing in Both Glucose-Excited and Glucose-Inhibited Central Neurons," *Diabetes*, vol. 51, no. 7, pp. 2056–2065, Jul. 2002.
- [481] U. Rajamani *et al.*, "Super-Obese Patient-Derived iPSC Hypothalamic Neurons Exhibit Obesogenic Signatures and Hormone Responses," *Cell Stem Cell*, vol. 22, no. 5, pp. 698–712.e9, 2018.
- [482] L. Wang *et al.*, "Differentiation of hypothalamic-like neurons from human pluripotent stem cells," *J. Clin. Invest.*, vol. 125, no. 2, pp. 796–808, Feb. 2015.
- [483] F. T. Merkle *et al.*, "Generation of neuropeptidergic hypothalamic neurons from human pluripotent stem cells," *Development*, vol. 142, no. 4, pp. 633–643, Feb. 2015.
- [484] K. Ogawa *et al.*, "Vasopressin-secreting neurons derived from human embryonic stem cells through specific induction of dorsal hypothalamic progenitors," *Sci. Rep.*, vol. 8, no. 1, p. 3615, Dec. 2018.
- [485] L. Lossi and A. Merighi, "The Use of ex Vivo Rodent Platforms in Neuroscience Translational Research With Attention to the 3Rs Philosophy," *Front. Vet. Sci.*, vol. 5, p. 164, 2018.
- [486] Q. Wei *et al.*, "Uneven balance of power between hypothalamic peptidergic neurons in the

- control of feeding," *Proc. Natl. Acad. Sci.*, vol. 115, no. 40, pp. E9489–E9498, Oct. 2018.
- [487] A. N. den Pol, C. Acuna, J. N. Davis, H. Huang, and X. Zhang, "Defining the caudal hypothalamic arcuate nucleus with a focus on anorexic excitatory neurons," *J. Physiol.*, vol. 597, no. 6, pp. 1605–1625, Mar. 2019.
- [488] S. Suyama, F. Maekawa, Y. Maejima, N. Kubota, T. Kadowaki, and T. Yada, "Glucose level determines excitatory or inhibitory effects of adiponectin on arcuate POMC neuron activity and feeding," *Sci. Rep.*, vol. 6, no. 1, p. 30796, Nov. 2016.
- [489] N. Chen *et al.*, "Direct modulation of GFAP-expressing glia in the arcuate nucleus bi-directionally regulates feeding," *Elife*, vol. 5, Oct. 2016.
- [490] J.-M. Israel, S. H. Oliet, and P. Ciofi, "Electrophysiology of Hypothalamic Magnocellular Neurons In vitro: A Rhythmic Drive in Organotypic Cultures and Acute Slices," *Front. Neurosci.*, vol. 10, Mar. 2016.
- [491] A. Kisner *et al.*, "Electrophysiological properties and projections of lateral hypothalamic parvalbumin positive neurons," *PLoS One*, vol. 13, no. 6, p. e0198991, Jun. 2018.
- [492] J. B. Regard, I. T. Sato, and S. R. Coughlin, "Anatomical profiling of G protein-coupled receptor expression," *Cell*, vol. 135, no. 3, pp. 561–71, Oct. 2008.
- [493] B. Li *et al.*, "A Comprehensive Mouse Transcriptomic BodyMap across 17 Tissues by RNA-seq," *Sci. Rep.*, vol. 7, no. 1, p. 4200, Dec. 2017.
- [494] D. Athauda *et al.*, "Exenatide once weekly versus placebo in Parkinson's disease: a randomised, double-blind, placebo-controlled trial," *Lancet*, vol. 390, no. 10103, pp. 1664–1675, Oct. 2017.
- [495] V. A. Gault and C. Hölscher, "GLP-1 receptor agonists show neuroprotective effects in animal models of diabetes," *Peptides*, vol. 100, pp. 101–107, 2018.
- [496] S. Carmo-Silva, C. Nobrega, L. Pereira de Almeida, and C. Cavadas, "Unraveling the Role of Ataxin-2 in Metabolism," *Trends Endocrinol. Metab.*, vol. 28, no. 4, pp. 309–318, Apr. 2017.
- [497] I. Lastres-Becker *et al.*, "Insulin receptor and lipid metabolism pathology in ataxin-2 knock-out mice," *Hum. Mol. Genet.*, vol. 17, no. 10, pp. 1465–1481, May 2008.
- [498] T.-R. Kiehl, A. Nechiporuk, K. P. Figueroa, M. T. Keating, D. P. Huynh, and S.-M. Pulst, "Generation and characterization of Sca2 (ataxin-2) knockout mice," *Biochem. Biophys. Res. Commun.*, vol. 339, no. 1, pp. 17–24, Jan. 2006.
- [499] D. R. Scoles, L. T. Pflieger, K. K. Thai, S. T. Hansen, W. Dansithong, and S.-M. Pulst, "ETS1 regulates the expression of ATXN2," *Hum. Mol. Genet.*, vol. 21, no. 23, pp. 5048–5065, Dec. 2012.
- [500] N. J. Lee *et al.*, "Osteoglycin, a novel coordinator of bone and glucose homeostasis," *Mol. Metab.*, vol. 13, pp. 30–44, Jul. 2018.
- [501] D. Benaki *et al.*, "Solution structure of humanin, a peptide against Alzheimer's disease-related neurotoxicity," *Biochem. Biophys. Res. Commun.*, vol. 329, no. 1, pp. 152–160, Apr. 2005.
- [502] K. Yen *et al.*, "Humanin Prevents Age-Related Cognitive Decline in Mice and is Associated with Improved Cognitive Age in Humans," *Sci. Rep.*, vol. 8, no. 1, p. 14212, Sep. 2018.
- [503] R. Kuliawat *et al.*, "Potent humanin analog increases glucose-stimulated insulin secretion through enhanced metabolism in the β cell," *FASEB J.*, vol. 27, no. 12, pp. 4890–4898, Dec. 2013.
- [504] Q. Qin *et al.*, "Humanin promotes mitochondrial biogenesis in pancreatic MIN6 β -cells," *Biochem. Biophys. Res. Commun.*, vol. 497, no. 1, pp. 292–297, 2018.
- [505] B. Schoenebeck, V. Bader, X. R. Zhu, B. Schmitz, H. Lübbert, and C. C. Stichel, "Sgk1, a cell survival response in neurodegenerative diseases," *Mol. Cell. Neurosci.*, vol. 30, no. 2, pp. 249–264, Oct. 2005.
- [506] H. Rangone *et al.*, "The serum- and glucocorticoid-induced kinase SGK inhibits mutant huntingtin-induced toxicity by phosphorylating serine 421 of huntingtin," *Eur. J. Neurosci.*, vol. 19, no. 2, pp. 273–279, Jan. 2004.
- [507] P. Miranda *et al.*, "The neuronal serum- and glucocorticoid-regulated kinase 1.1 reduces neuronal excitability and protects against seizures through upregulation of the M-current," *J. Neurosci.*, vol. 33, no. 6, pp. 2684–96, Feb. 2013.
- [508] J. Jourquin *et al.*, "Tissue inhibitor of metalloproteinases-1 (TIMP-1) modulates neuronal death, axonal plasticity, and learning and memory," *Eur. J. Neurosci.*, vol. 22, no. 10, pp. 2569–78, Nov. 2005.
- [509] Ashutosh, C. Chao, K. Borgmann, K. Brew, and A. Ghorpade, "Tissue inhibitor of metalloproteinases-1 protects human neurons from staurosporine and HIV-1-induced apoptosis: mechanisms and relevance to HIV-1-associated dementia," *Cell Death Dis.*, vol. 3, p. e332, Jun. 2012.

- [510] L. Zhan, L. Zheng, T. Hosoi, Y. Okuma, and Y. Nomura, "Stress-Induced Neuroprotective Effects of Epiregulin and Amphiregulin," *PLoS One*, vol. 10, no. 2, p. e0118280, Feb. 2015.
- [511] Y. Liang *et al.*, "Thioredoxin-interacting protein mediates mitochondrion-dependent apoptosis in early brain injury after subarachnoid hemorrhage," *Mol. Cell. Biochem.*, vol. 450, no. 1–2, pp. 149–158, Jan. 2019.
- [512] C.-J. Su *et al.*, "Thioredoxin-interacting protein induced α -synuclein accumulation via inhibition of autophagic flux: Implications for Parkinson's disease," *CNS Neurosci. Ther.*, vol. 23, no. 9, pp. 717–723, Sep. 2017.
- [513] C.-C. Liu, C.-C. Liu, T. Kanekiyo, H. Xu, and G. Bu, "Apolipoprotein E and Alzheimer disease: risk, mechanisms and therapy," *Nat. Rev. Neurol.*, vol. 9, no. 2, pp. 106–18, 2013.
- [514] J. Muffat and D. W. Walker, "Apolipoprotein D: An overview of its role in aging and age-related diseases," *Cell Cycle*, vol. 9, no. 2, pp. 269–273, Jan. 2010.
- [515] S. Ramesh, M. Govindarajulu, V. Suppiramaniam, T. Moore, and M. Dhanasekaran, "Autotaxin–Lysophosphatidic Acid Signaling in Alzheimer's Disease," *Int. J. Mol. Sci.*, vol. 19, no. 7, p. 1827, Jun. 2018.
- [516] J. Zhao *et al.*, "Neuroinflammation induced by lipopolysaccharide causes cognitive impairment in mice," *Sci. Rep.*, vol. 9, no. 1, p. 5790, Apr. 2019.
- [517] C. R. A. Batista, G. F. Gomes, E. Candelario-Jalil, B. L. Fiebich, and A. C. P. de Oliveira, "Lipopolysaccharide-Induced Neuroinflammation as a Bridge to Understand Neurodegeneration," *Int. J. Mol. Sci.*, vol. 20, no. 9, p. 2293, May 2019.
- [518] J. Nakai, M. Ohkura, and K. Imoto, "A high signal-to-noise Ca^{2+} probe composed of a single green fluorescent protein," *Nat. Biotechnol.*, vol. 19, no. 2, pp. 137–141, Feb. 2001.
- [519] M. K. Holt, I. J. Llewellyn-Smith, F. Reimann, F. M. Gribble, and S. Trapp, "Serotonergic modulation of the activity of GLP-1 producing neurons in the nucleus of the solitary tract in mouse," *Mol. Metab.*, vol. 6, no. 8, pp. 909–921, 2017.
- [520] N. A. Steinmetz *et al.*, "Aberrant Cortical Activity in Multiple GCaMP6-Expressing Transgenic Mouse Lines," *eNeuro*, vol. 4, no. 5.
- [521] Y. Yang *et al.*, "Improved calcium sensor GCaMP-X overcomes the calcium channel perturbations induced by the calmodulin in GCaMP," *Nat. Commun.*, vol. 9, no. 1, p. 1504, Dec. 2018.
- [522] C. Cabou *et al.*, "Brain Glucagon-Like Peptide-1 Regulates Arterial Blood Flow, Heart Rate, and Insulin Sensitivity," *Diabetes*, vol. 57, no. 10, pp. 2577–2587, Oct. 2008.
- [523] C. Knauf, "Brain glucagon-like peptide-1 increases insulin secretion and muscle insulin resistance to favor hepatic glycogen storage," *J. Clin. Invest.*, vol. 115, no. 12, pp. 3554–3563, Dec. 2005.
- [524] W. M. Pardridge, "CSF, blood-brain barrier, and brain drug delivery," *Expert Opin. Drug Deliv.*, vol. 13, no. 7, pp. 963–75, 2016.
- [525] Y. Iwasaki *et al.*, "GLP-1 release and vagal afferent activation mediate the beneficial metabolic and chronotherapeutic effects of D-allulose," *Nat. Commun.*, vol. 9, no. 1, p. 113, Dec. 2018.
- [526] D. Chen *et al.*, "A nonpeptidic agonist of glucagon-like peptide 1 receptors with efficacy in diabetic db/db mice," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 104, no. 3, pp. 943–8, Jan. 2007.
- [527] J. M. Tarasoff-Conway *et al.*, "Clearance systems in the brain-implications for Alzheimer disease," *Nat. Rev. Neurol.*, vol. 11, no. 8, pp. 457–70, Aug. 2015.
- [528] M. Rizk, L. Zou, R. Savic, and K. Dooley, "Importance of Drug Pharmacokinetics at the Site of Action," *Clin. Transl. Sci.*, vol. 10, no. 3, pp. 133–142, May 2017.
- [529] D. Karra and R. Dahm, "Transfection Techniques for Neuronal Cells," *J. Neurosci.*, vol. 30, no. 18, pp. 6171–6177, May 2010.
- [530] L. Onfroy *et al.*, "G protein stoichiometry dictates biased agonism through distinct receptor-G protein partitioning," *Sci. Rep.*, vol. 7, no. 1, p. 7885, Dec. 2017.
- [531] J. Naylor *et al.*, "Use of CRISPR/Cas9-engineered INS-1 pancreatic β cells to define the pharmacology of dual GIPR/GLP-1R agonists," *Biochem. J.*, vol. 473, no. 18, pp. 2881–91, 2016.
- [532] T. Buenaventura *et al.*, "Agonist binding affinity determines palmitoylation of the glucagon-like peptide-1 receptor and its functional interaction with plasma membrane nanodomains in pancreatic beta cells," 2018.
- [533] S. Curry, "Lessons from the crystallographic analysis of small molecule binding to human serum albumin," *Drug Metab. Pharmacokinet.*, vol. 24, no. 4, pp. 342–57, 2009.
- [534] C. Chaudhury *et al.*, "The major histocompatibility complex-related Fc receptor for IgG (FcRn) binds albumin and prolongs its lifespan," *J. Exp. Med.*, vol. 197, no. 3, pp. 315–22, Feb. 2003.
- [535] C. L. Anderson, C. Chaudhury, J. Kim, C. L. Bronson, M. A. Wani, and S. Mohanty,

- "Perspective-- FcRn transports albumin: relevance to immunology and medicine.," *Trends Immunol.*, vol. 27, no. 7, pp. 343–8, Jul. 2006.
- [536] D. K. Clodfelter *et al.*, "Effects of non-covalent self-association on the subcutaneous absorption of a therapeutic peptide.," *Pharm. Res.*, vol. 15, no. 2, pp. 254–62, Feb. 1998.
- [537] T. Heise, L. Nosek, S. G. Böttcher, H. Hastrup, and H. Haahr, "Ultra-long-acting insulin degludec has a flat and stable glucose-lowering effect in type 2 diabetes.," *Diabetes. Obes. Metab.*, vol. 14, no. 10, pp. 944–50, Oct. 2012.
- [538] L. B. Knudsen *et al.*, "Potent Derivatives of Glucagon-like Peptide-1 with Pharmacokinetic Properties Suitable for Once Daily Administration," *J. Med. Chem.*, vol. 43, no. 9, pp. 1664–1669, May 2000.
- [539] K. Adelhorst, B. B. Hedegaard, L. B. Knudsen, and O. Kirk, "Structure-activity studies of glucagon-like peptide-1.," *J. Biol. Chem.*, vol. 269, no. 9, pp. 6275–8, Mar. 1994.
- [540] K. Madsen *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.*, vol. 50, no. 24, pp. 6126–6132, Nov. 2007.
- [541] J. Lau *et al.*, "Discovery of the Once-Weekly Glucagon-Like Peptide-1 (GLP-1) Analogue Semaglutide.," *J. Med. Chem.*, vol. 58, no. 18, pp. 7370–80, Sep. 2015.
- [542] E. L. Stahl, L. Zhou, F. J. Ehler, and L. M. Bohn, "A novel method for analyzing extremely biased agonism at G protein-coupled receptors.," *Mol. Pharmacol.*, vol. 87, no. 5, pp. 866–77, May 2015.
- [543] M. Cottet *et al.*, "BRET and Time-resolved FRET strategy to study GPCR oligomerization: from cell lines toward native tissues," *Front. Endocrinol. (Lausanne)*, vol. 3, 2012.
- [544] H. J. Motulsky and L. C. Mahan, "The kinetics of competitive radioligand binding predicted by the law of mass action.," *Mol. Pharmacol.*, vol. 25, no. 1, pp. 1–9, Jan. 1984.
- [545] C. Widmann, W. Dolci, and B. Thorens, "Agonist-induced internalization and recycling of the glucagon-like peptide-1 receptor in transfected fibroblasts and in insulinomas.," *Biochem. J.*, vol. 310 (Pt 1, pp. 203–14, Aug. 1995.
- [546] B. P. Ward *et al.*, "Peptide lipidation stabilizes structure to enhance biological function," *Mol. Metab.*, vol. 2, no. 4, pp. 468–479, Nov. 2013.
- [547] E. Staes *et al.*, "Acylated and unacylated ghrelin binding to membranes and to ghrelin receptor: Towards a better understanding of the underlying mechanisms," *Biochim. Biophys. Acta - Biomembr.*, vol. 1798, no. 11, pp. 2102–2113, Nov. 2010.
- [548] S. Rajan *et al.*, "Chronic hyperglycemia downregulates GLP-1 receptor signaling in pancreatic β -cells via protein kinase A.," *Mol. Metab.*, vol. 4, no. 4, pp. 265–76, Apr. 2015.
- [549] E. Boslem, P. J. Meikle, and T. J. Biden, "Roles of ceramide and sphingolipids in pancreatic β -cell function and dysfunction.," *Islets*, vol. 4, no. 3, pp. 177–87.
- [550] D. J. Drucker, "Mechanisms of Action and Therapeutic Application of Glucagon-like Peptide-1," *Cell Metab.*, vol. 27, no. 4, pp. 740–756, Apr. 2018.
- [551] J. van Can, B. Sloth, C. B. Jensen, A. Flint, E. E. Blaak, and W. H. M. Saris, "Effects of the once-daily GLP-1 analog liraglutide on gastric emptying, glycemic parameters, appetite and energy metabolism in obese, non-diabetic adults.," *Int. J. Obes. (Lond)*, vol. 38, no. 6, pp. 784–93, Jun. 2014.
- [552] C. E. Pritchett and A. Hajnal, "Glucagon-like peptide-1 regulation of carbohydrate intake is differentially affected by obesogenic diets.," *Obesity (Silver Spring)*, vol. 20, no. 2, pp. 313–7, Feb. 2012.
- [553] F. A. Duca, Y. Sakar, and M. Covasa, "Combination of obesity and high-fat feeding diminishes sensitivity to GLP-1R agonist exendin-4.," *Diabetes*, vol. 62, no. 7, pp. 2410–5, Jul. 2013.
- [554] J. S. Ten Kulve *et al.*, "Decreased Hypothalamic Glucagon-Like Peptide-1 Receptor Expression in Type 2 Diabetes Patients.," *J. Clin. Endocrinol. Metab.*, vol. 101, no. 5, pp. 2122–9, 2016.
- [555] G. Levy, "Pharmacologic target-mediated drug disposition," *Clin. Pharmacol. Ther.*, vol. 56, no. 3, pp. 248–252, Sep. 1994.
- [556] B.-F. Krippendorff, K. Kuester, C. Kloft, and W. Huisinga, "Nonlinear pharmacokinetics of therapeutic proteins resulting from receptor mediated endocytosis.," *J. Pharmacokinet. Pharmacodyn.*, vol. 36, no. 3, pp. 239–60, Jun. 2009.
- [557] D. Parkes *et al.*, "Pharmacokinetic actions of exendin-4 in the rat: Comparison with glucagon-like peptide-1," *Drug Dev. Res.*, vol. 53, no. 4, pp. 260–267, Aug. 2001.
- [558] W. Gao and W. J. Jusko, "Pharmacokinetic and pharmacodynamic modeling of exendin-4 in type 2 diabetic Goto-Kakizaki rats.," *J. Pharmacol. Exp. Ther.*, vol. 336, no. 3, pp. 881–90, Mar. 2011.

- [559] W. Gao and W. J. Jusko, "Target-mediated pharmacokinetic and pharmacodynamic model of exendin-4 in rats, monkeys, and humans.," *Drug Metab. Dispos.*, vol. 40, no. 5, pp. 990–7, May 2012.
- [560] L. K. Henchey, A. L. Jochim, and P. S. Arora, "Contemporary strategies for the stabilization of peptides in the alpha-helical conformation.," *Curr. Opin. Chem. Biol.*, vol. 12, no. 6, pp. 692–7, Dec. 2008.
- [561] B. P. Ward *et al.*, "Peptide lipidation stabilizes structure to enhance biological function.," *Mol. Metab.*, vol. 2, no. 4, pp. 468–79, 2013.
- [562] D. F. Sargent and R. Schwyzer, "Membrane lipid phase as catalyst for peptide-receptor interactions.," *Proc. Natl. Acad. Sci.*, vol. 83, no. 16, pp. 5774–5778, Aug. 1986.
- [563] B. Chen, Y. Sun, J. Niu, G. K. Jarugumilli, and X. Wu, "Protein Lipidation in Cell Signaling and Diseases: Function, Regulation, and Therapeutic Opportunities," *Cell Chem. Biol.*, vol. 25, no. 7, pp. 817–831, Jul. 2018.
- [564] P. Zhang, L. Covic, and A. Kuliopulos, "Pepducins and Other Lipidated Peptides as Mechanistic Probes and Therapeutics.," *Methods Mol. Biol.*, vol. 1324, pp. 191–203, 2015.
- [565] C. Gauna, B. van de Zande, A. van Kerkwijk, A. P. N. Themmen, A. J. van der Lely, and P. J. D. Delhanty, "Unacylated ghrelin is not a functional antagonist but a full agonist of the type 1a growth hormone secretagogue receptor (GHS-R)," *Mol. Cell. Endocrinol.*, vol. 274, no. 1–2, pp. 30–34, Aug. 2007.
- [566] A. Edwards and A. Abizaid, "Clarifying the Ghrelin System's Ability to Regulate Feeding Behaviours Despite Enigmatic Spatial Separation of the GHSR and Its Endogenous Ligand.," *Int. J. Mol. Sci.*, vol. 18, no. 4, Apr. 2017.
- [567] NCD Risk Factor Collaboration (NCD-RisC), "Trends in adult body-mass index in 200 countries from 1975 to 2014: a pooled analysis of 1698 population-based measurement studies with 19·2 million participants.," *Lancet (London, England)*, vol. 387, no. 10026, pp. 1377–1396, Apr. 2016.
- [568] M. A. Bethel *et al.*, "Cardiovascular outcomes with glucagon-like peptide-1 receptor agonists in patients with type 2 diabetes: a meta-analysis.," *lancet. Diabetes Endocrinol.*, vol. 6, no. 2, pp. 105–113, 2018.
- [569] J. F. E. Mann *et al.*, "Liraglutide and Renal Outcomes in Type 2 Diabetes," *N. Engl. J. Med.*, vol. 377, no. 9, pp. 839–848, Aug. 2017.
- [570] K. Bettge, M. Kahle, M. S. Abd El Aziz, J. J. Meier, and M. A. Nauck, "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.*, vol. 19, no. 3, pp. 336–347, 2017.
- [571] O. G. Kolterman *et al.*, "Pharmacokinetics, pharmacodynamics, and safety of exenatide in patients with type 2 diabetes mellitus.," *Am. J. Health. Syst. Pharm.*, vol. 62, no. 2, pp. 173–81, Jan. 2005.
- [572] M. A. Nauck *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*, vol. 39, no. 2, pp. 231–41, Feb. 2016.
- [573] M. Kleinert, P. Kotzbeck, T. Altendorfer-Kroath, T. Birngruber, M. H. Tschöp, and C. Clemmensen, "Time-resolved hypothalamic open flow micro-perfusion reveals normal leptin transport across the blood-brain barrier in leptin resistant mice.," *Mol. Metab.*, vol. 13, pp. 77–82, 2018.
- [574] T. Birngruber *et al.*, "Cerebral open flow microperfusion: A new in vivo technique for continuous measurement of substance transport across the intact blood-brain barrier," *Clin. Exp. Pharmacol. Physiol.*, vol. 40, no. 12, pp. 864–871, Dec. 2013.
- [575] D. Liu *et al.*, "Quantitative determination of unbound levofloxacin by simultaneous microdialysis in rat pancreas after intravenous and oral doses.," *J. Pharm. Pharmacol.*, vol. 66, no. 9, pp. 1215–21, Sep. 2014.
- [576] K. Hunter and C. Hölscher, "Drugs developed to treat diabetes, liraglutide and lixisenatide, cross the blood brain barrier and enhance neurogenesis.," *BMC Neurosci.*, vol. 13, p. 33, Mar. 2012.