

IMPERIAL

**Unravelling the Significance of the Human GLP-1
Receptor rs10305492 Variant: Implications for
Metabolic Health and Personalised Therapeutics**

by

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Declaration of authenticity and copyright

I, Liliane El Eid, declare that the work presented in this thesis is my own, and that no part of the dissertation has been submitted for any other degree. Any work carried out by others has been acknowledged and appropriately referenced below:

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"This world is indeed a living being endowed with a soul and intelligence ... a single visible living entity containing all other living entities, which by their nature are all related." Plato

Abstract

Glucagon-like peptide-1 receptor (GLP-1R) agonists are a highly effective class of therapies for type 2 diabetes (T2D), yet there are variable patient responses. Genomic variation in the human *Glp1r* altering structure, signal transduction and function, might be directly linked to therapeutic responses in patients. A naturally occurring low-frequency gain-of-function (GoF) missense variant, rs10305492 G>A (A316T), enriched in non-Finish Europeans, protects against T2D and cardiovascular disease. To examine the association between this mutation and glucose homeostasis, a knock-in mouse model bearing the homozygous A316T substitution in the human GLP-1R was made using CRISPR-Cas9 technology. These *hGlp1r^{A316T/A316T}* mice displayed lower fasting blood glucose levels with improved glucose tolerance, plasma insulin, and insulin secretion, even under metabolic stress. They also exhibited alterations in islet cytoarchitecture and β -cell identity, indicative of a compensatory mechanism under high-fat, high sucrose diet (HFHSD) challenge. Across all models investigated, the A316T variant exhibited characteristics of constitutive activation, including increased basal trafficking and signalling, with blunted incretin-induced responses which were more pronounced for certain GLP-1R agonists (GLP-1RA). This was further supported by structural insights from cryo-EM and computational analyses of the A316T compared to the WT GLP-1R. In conclusion, our study suggests that GLP-1R variants like the rs10305492 determine pharmacological responses to GLP-1R-targeting anti-diabetic therapies. This

highlights the importance of precise molecular characterisation of GLP-1R genetic variants to predict individual responses to specific GLP-1R therapies and improved drug target validation techniques.

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Abbreviations

2-Mono-Acylglycerol (2-MAG)

Adenosine Triphosphate (ATP)

Adenylate Cyclase (AC)

Adenylate Cyclase-Activating Peptide Receptor (PAC1)

Affinity Purification and Mass Spectrometry (AP-MS)

American Diabetes Association (ADA)

Amylin Receptor (AMYR)

And Immunohistochemistry (IHC)

Arcuate Nucleus (ARC)

Area Under the Curve (AUC)

ATP-Sensitive K⁺ (KATP)

ATP-Sensitive K⁺ Channels (KATP-Channels),

Bafilomycin A1 (BAF)

Bioluminescence Resonance Energy Transfer (BRET)

Body Mass Index (BMI)

Bovine Serum Albumin (BSA)

Ca²⁺ Sensing Receptor (CASR)

Calcitonin Receptor (CALCR)

Calcitonin Receptor (CALCR)

Camp Response Element (CREPG)

Chinese Hamster Ovary (CHO)

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)

CMV (Cytomegalovirus)

Constitutively Activating Mutations (CAMs)

Cryo-Electron Microscopy (Cryo-EM)

Cyclic Adenosine-Monophosphate (cAMP)

Detergent Resistant Membrane Fraction (DRF)

Detergent Soluble Membrane Fraction (DSF)

Diabetes Mellitus (DM)

Diacylglycerol (DAG)

Dipeptidyl Peptidase-4 (DPP-4)

Donor Single-Stranded Oligo DNA Nucleotides (SSODN)

Double Strand Breaks (DSBs)

Endoplasmic Reticulum (ER)

Endosomal Sorting Complex Required for Transport (ESCRT)

Extracellular Domain (ECD)

Extracellular Loop (ECL)

Extracellular Matrix (ECM)

Fasting Plasma Glucose (FPG)

Follicle Stimulating Hormone (FSH)

Follicle Stimulating Hormone Receptor (FSHR)

Forskolin (FSK)

Free Fatty Acid Receptor (FFAR)

FRET (Fluorescence Resonance Energy Transfer)

G Protein Coupled Receptors (GPCRs)

Gain Of Function Variants (GoF)

Gastric Inhibitory Polypeptide Receptor (GIPR)

Gastrointestinal Mediated Glucose Disposal (GIGD)

Genome Wide Association Studies (GWAS)

Genomic DNA (gDNA)

Gestational Diabetes Mellitus (GDM)

Glicentin-Related Pancreatic Polypeptide (GRPP)

Glucagon Like-Peptide 1 and 2 Receptor (GLP-1R and GLP-2R)

Glucagon Receptor (GCGR)

Glucagon-Like Peptide-1 (GLP-1)

Glucokinase Activators (GKAs)

Glucose-Dependent Insulinotropic Peptide (GIP)

Glucose-Sensing (GS)

Glucose-Stimulated Insulin Secretion (GSIS)

High –Fat, High Sucrose Diet (HFHSD)

Homogeneous Time-Resolved Fluorescence (HTRF)

Homologous End Joining (NHEJ)

Homology Directed-Repair (HDR)

Human Embryonic Stem Cells (hESCs)

Human Induced Pluripotent Stem Cells (hiPSCs)

Human Pluripotent Stem Cells (hPSCs)

Hydrogen Bond (HB)

Inhibitor Isobutyl Methylxanthine (IBMX)

Insulin Receptor (IR)

Insulin Receptor Substrate (IRS)

Insulin Resistance (IR)

International Diabetes Federation (IDF)

International Union of Basic and Clinical Pharmacology (IUPHAR)

Intracellular Loop (ICL)

Intraperitoneal Glucose Tolerance Test (IPGTT)

Islet Amyloid Polypeptide (IAPP Or Amylin)

Label-Free Quantification (LFQ)

Latent Autoimmune Diabetes in Adults (LADA)

Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)

Loss Of Function Variants (LOF)

Magnesium Adenosine Diphosphate (Mg-ADP)

Maturity Onset Diabetes of The Young (MODY)

Membrane Contact Sites (MCS)

Minor Allele Frequency (MAF)

Mitogen Activated Protein Kinase (MAPK)

Molecular Dynamics (MD)

Next Generation Sequencing (NGS)

Nucleus Tractus Solitarii (NTS)

Oral Glucose Tolerance Test (OGTT)

Pancreatic Polypeptide (PP)

Paraformaldehyde (PFA)

Parathyroid Hormone Receptor (PTHr1 And 2)

Peptide Transporter 1 (PEPT1)

Peptidyl-Glycine Alpha-Amidating Monooxygenase (PAM)

Phosphokinase C (PKC)

Phospholipase C (PLC)

Phosphotyrosine Binding (PTB)

Pleckstrin Homology (PH)

Polycystic Ovary Syndrome (PCOS)

Positive Allosteric Mediator (PAM)

Proglucagon Fragment (MPGF)

Prohormone Convertases (PC/PCSK)

Protein Data Bank (PDB)

Protein Kinase (PKA)

Quantitative PCR (qPCR)

Raster Image Correlation Spectroscopy (RICS)

Region Of Interest (ROI)

Ribonucleoprotein (RNP)

Root Mean Square Fluctuation (RMSF)

Roux-En-Y Gastric Bypass (RYGB)

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Secretin Receptor (SECR)

Serum-Free Medium (SFM)

Single Nucleotide Polymorphism (SNP)

Single-Guide RNA (sgRNA)

Sodium N-(8-(2-Hydroxybenzoyl) Amino) Caprylat (SNAC)

Soluble N-Ethylmaleimide-Sensitive Factor Attachment Protein Receptor Proteins (SNAREs)

Sorting Nexin-27 (SNX27)

Splice Variants (SVs)

Stem-Cell Derived Islets (SC-Islets)

Study To Understand the Genetics of Acute Responses to Metformin and Glipizide in Humans (SUGAR-MGH)

Sulfonylurea Receptors (SUR)

Tetramethylrhodamine (TMR)

Time-Resolved Fluorescence Resonance Energy Transfer (TR-FRET)

Total Internal Reflection Fluorescence (TIRF)

Trans-Golgi Network (TGN)

Transmembrane (TM)

Transmembrane Domain (TMD)

Type 1 Diabetes (T1D)

Type 2 Diabetes (T2D)

VAMP Associated Protein B (VAPB)

Vasoactive Intestinal Peptide Receptor (VIP1 And 2)

Voltage Dependent Calcium Channels (VDC)

Voltage-Dependent Ca^{2+} Channels (VDCCs)

Wild Type (WT)

Chapter 1: General Introduction

1.1 Diabetes and obesity

1.1.1 Diabetes Mellitus: a global health problem

The diabetes pandemic is a substantial public health issue, with the International Diabetes Federation (IDF) estimated 537 million people living with diabetes worldwide, and this number forecast to reach 1.3 billion people by 2050 (1).

The NICE guidelines define diabetes mellitus (DM) as a group of complex metabolic disorders characterised by chronic hyperglycaemia (random plasma glucose of $>11.1\text{mmol/L}$) resulting from impaired insulin secretion, insulin resistance, or both (2).

Currently, major diabetes organisations such as the American Diabetes Association (ADA) classify diabetes into four different groups: type 1 diabetes (T1D), type 2 diabetes (T2D), gestational diabetes mellitus (GDM), and specific types of diabetes due to other causes including monogenic diabetes, diseases of the exocrine pancreas (such as cystic fibrosis), and drug or chemical-induced diabetes. Whilst T1D, T2D and GDM are the most common types of diabetes, about 2% of people have other types of diabetes (3). Research is increasingly investigating mechanisms underlying these rare forms of diabetes. Monogenic diabetes includes conditions such as maturity onset diabetes of the young (MODY) and neonatal diabetes (which develops in babies), whereby diabetes is caused by single gene mutations in the hepatocyte nuclear factor-1-alpha (*Hnf1a*, *Hnf1b*, *Hnf4a*, glucokinase (*Gck*) or *Kcnj11*, *Abcc8* and *Ins* genes, respectively (4, 5). Alternatively, it also comprises rare genetically

inherited diabetes-associated syndromes such as Wolfram, Alström, Wolcott Rallison and Mitchell-Riley syndromes (4). Other types of diabetes are associated with pancreatic damage as a result of autoimmune pathogenesis (latent autoimmune diabetes in adults (LADA) (6) or pancreatic inflammatory diseases such as pancreatitis, pancreatic cancer, cystic fibrosis and hemochromatosis (pancreatogenic/type 3c diabetes) (7). Certain medications such as antipsychotics and steroids could also lead to diabetes (8).

Most cases (96%) of DM cases are attributable to T2D, which has a strong association with obesity, lack of exercise, and poor diet (1). In the UK, new statistics published by Diabetes UK show that more than 6 million people are now living with a diabetes diagnosis or are at a high risk of developing T2D (9, 10). Moreover, patients with diabetes are also at risk of comorbidities including cardiovascular disease, nephropathy, retinopathy, and neuropathy (11). Expectedly, T2D was also deemed one of the top 10 causes of death and disability in the UK (9, 10, 12).

1.1.2 Obesity and its link to diabetes

Obesity, another metabolic disorder, is defined as having a body mass index (BMI) of 30 or higher, whilst a BMI of 25.0-29.9 is classified as overweight (13). A person's BMI is calculated by dividing an individual's weight in kilograms by the square of their height in meters. The prevalence of obesity has been rapidly growing globally, with estimates that 25.9% and 37.9% of adults in England are obese and overweight, respectively (14). Therefore, with one of the highest obesity rates in Europe, tackling obesity is one of the greatest long-term health challenges and concerns faced by the UK. Obesity results from an energy imbalance, where calorie intake exceeds energy expenditure, leading to an accumulation of excess body fat and

adipose tissue dysfunction (15). This dysfunction results in adverse metabolic effects, including insulin resistance, dyslipidaemia, and chronic inflammation, which are all risk factors for the development of T2D and other associated comorbidities (16, 17). Indeed, obesity is estimated to increase the risk of developing T2D by seven times compared to individuals of healthy weight (18). Moreover, collective evidence from previous studies suggests that obesity management measures such as bariatric surgery, coupled with lifestyle modifications such as increased physical activity (19), play a crucial role in improving insulin sensitivity, and enhancing glucose control through calorie restriction, nutrient deviation, and reduction of adipose tissue in individuals with 'diabesity' (20-22). Yet, there are multiple factors influencing obesity onset attributable to the complex interplay of genetic, environmental, and behavioural components.

1.1.3 Type 2 Diabetes (T2D)

T2D is characterised by deficient insulin secretion by pancreatic β -cells, insulin resistance (IR) in peripheral tissues (skeletal muscle, liver, and adipose tissue), or a combination of both, resulting in dysregulation of carbohydrate, lipid, and protein metabolism.

Early studies by DeFronzo in 1987 established pancreatic β -cell failure and IR in muscle and liver, also known as the “triumvirate”, as the core defects of T2D (23). The development of T2D is triggered when individuals with a genetic or epigenetic predisposition to T2D experience chronic elevated glucose levels (hyperglycaemia), due to IR triggered by environmental factors such as obesity and physical inactivity. IR is defined as an impaired glucose uptake response to insulin in insulin-sensitive peripheral tissues such as the liver, muscle, and adipose tissue.

Typically, T2D is preceded by pre-diabetes, an intermediate stage between normal glucose regulation and overt diabetes, defined by a fasting glucose level of 5.5-6.9 mmol/L and impaired glucose tolerance met by 2-hour post glucose ingestion levels between 7.8-11 mmol/L. Pre-diabetes is estimated to affect around 12% of adults in England (~5.1 million), with ~5-10% progressing to T2D each year (10). Interestingly, of those South Asian or mixed-race individuals and people with social deprivation have an increased risk of progression from pre-diabetes to T2D. Additionally, there is growing clinical evidence to suggest that management at this stage with effective lifestyle (calorie restriction, increased physical activity, and support for psychological obstacles) or pharmacological intervention (including metformin or GLP-1 mimetics) can significantly delay or prevent progression to T2D, or overt diabetes. Yet, if proper action is not taken, glucose tolerance is further impaired and β -cells will completely fail to compensate for the maximal insulin resistance, resulting in fasting glucose levels exceeding 7.8 mmol/L, ultimately leading to a diagnosis of T2D (24).

1.1.4 Risk factors and pathogenesis of T2D

T2D diagnosis is complicated by the fact that T2D progression is slow and asymptomatic, remaining largely undiagnosed until the appearance of classic symptoms of advanced stages of the disease such as severe hyperglycaemia, weight loss, blurred vision, polyuria, and polydipsia (25). The pathogenesis/aetiology of T2D involves multiple complex and interrelated pathways resulting from a combination of polygenic and strong environmental factors. Studies on T2D risk factors report that the major associated factors are family history (genetic), ethnic background, increasing age, and lifestyle factors such as obesity, poor diet, and physical inactivity (26).

The first multi-organ hypothesis of T2D pathogenesis was introduced by the physician Rafael DeFronzo in 1987, where he utilised experiments and clinical studies to present the “triumvirate”, proposing β -cell dysfunction in the pancreas and insulin resistance in the muscle and liver as contributors to T2D pathophysiology (23, 27). β -cell dysfunction is defined as the failure of β -cells to synthesise and secrete enough insulin, leading to a build-up of glucose in the bloodstream (hyperglycaemia). Whereas insulin resistance (IR) is characterised by a decreased responsiveness to insulin in peripheral tissue and thus an inability to reduce glucose levels in the blood. Upon glucose intolerance, β -cells initially respond to IR induced hyperglycaemia with a compensatory increase in insulin secretion through β -cell hyperplasia and enhanced insulin biosynthesis, to maintain normoglycaemia (28). However, chronically elevated glucose (glucotoxicity) and lipids (lipotoxicity) levels eventually lead to β -cell exhaustion and dysfunction, characterized by ER stress-induced impairments in proinsulin processing and insulin secretion, increased oxidative stress, mitochondrial dysfunction (29), and inflammation, culminating in increased β -cell apoptosis and dedifferentiation (30-32). Progressive loss of functional β -cell mass subsequently reduces insulin secretion capacity, aggravating IR further due to prolonged hyperglycaemia, thus perpetuating the cycle and exacerbating disease progression (33).

In 2009, DeFronzo added five additional organ specific pathways contributing to glucose intolerance and hyperglycaemic state, forming the “ominous octet” (34). These five pathways were adipose tissue dysfunction resulting in lipotoxicity, impaired incretin effect, pancreatic α -cell dysfunction resulting in elevated glucagon secretion, increased glucose reabsorption in the kidneys due elevated SGLT2 levels, and impaired appetite regulation by hypothalamus in the brain (34, 35).

Despite researchers believing that the ominous octet forms the core of T2D pathophysiology, further experiments proposed additional pathways that contributed in a minor yet significant manner. These include the recently proposed “sweetening sixteen”, with the addition of eight additional pathways: vitamin D deficiency, altered catecholamine and monoamine neurotransmitter levels in the brain (increased serotonin and reduced dopamine levels), reduction of synchronised hypothalamus mediated melatonin release, iron overload, increased intestinal glucose absorption by SGLT1, gut dysbiosis, testosterone deficiency, renin angiotensin system, and impaired suppression of renal gluconeogenesis (Figure 1.1) (36).

To date, over 700 loci have been associated to T2D risk in genome wide association studies (GWAS) (37). As T2D is a complex polygenic disease, single gene contributions are minor but together they play a significant role in increasing the risk of developing the disease. Most major T2D risk alleles identified by GWAS are in genes directly involved in β -cell function and insulin secretion (38). Notable examples include polymorphisms in *Tcf7l2*, encoding a transcription factor and master regulator critical for proinsulin production and processing (39, 40), and *SLC30A8*, encoding the ZnT8 zinc transporter essential for proper insulin storage and secretion from β -cell secretory granules (41). Additional risk alleles have been identified in genes involved in insulin secretion regulation such as *Abcc8*, *Gcgr*, *Capn10*, and those involved in glucose sensing and glucose transport, including *Glut2*, *Gcgr*, *Capn10* (37, 42).

Additionally, T2D occurs more frequently in individuals belonging to certain ethnic groups such as South Asian, African, Caribbean, Arab, and other ethnic backgrounds in the UK (25). Family history is thus a strong explanation for the pathogenesis of T2D in these ethnic groups, yet these genetic factors are complex and remain largely uncharacterised. Further, unlike T1D, no immune response and autoimmunity genes have been associated with pancreatic β -cell

dysfunction in T2D (25). Additional low-frequency rare variants have been recently associated with glycaemic traits and T2D risk in large-cohort GWAS studies (*Ccnd2*, *Pam*, *Pdx1*, *Kcnj11*, *G6pc2*, *Glp1r*, *Gipr*, and *Glp2r*) (37, 42-50).

Although T2D is recognised as an age-related disease, the mechanisms linking diabetes and aging are still unclear. In 2009, work by Minamino *et al.* reported novel insights into the link between aging in adipose tissue and diabetes (51). The study proposed that aging associated with telomere shortening, initiating an increase in tumour suppressor p53 protein levels along with other senescence markers in adipose tissue (β -galactosidase and *Cdkn1a* mRNA), resulting in increased inflammation, insulin resistance and impaired glucose tolerance in mice (51).

Lifestyle factors such as poor diet and lack of physical exercise also play a crucial role in the development of T2D. Obesity as mentioned above, particularly excess visceral abdominal fat, is a major risk factor, as it promotes insulin resistance. The ADA has recommended adoption of a diabetic diet and improved eating practices as a key management strategy for T2D (52). A large and growing body of literature has investigated the link between poor dietary choices and metabolic diseases such as diabetes and obesity (53). Data from these studies suggest that the composition, quality, and volume of food are all factors strongly linked to obesity and diabetes. Whilst high intake of red meat, sweets, saturated fats, and soft drinks (high fructose) contribute to increased risk of T2D (52, 53). Consumption of a fruit and vegetable-based diet such as the mediterranean diet, rich in nutrients, fibre and antioxidants, protects against T2D development (54). In the same vein, it is well established that increasing physical activity plays an important role in reducing the risk of diabetes and obesity (55, 56). Several cross-sectional and longitudinal studies have found an association with sedentary lifestyles and behaviours

with an increased risk of diabetes, obesity, and cardiovascular disease (57-59). On the other hand, even mild physical activity increasing total energy expenditure is associated with improved insulin sensitivity because of enhanced glucose transport and uptake in muscle as well as a reduction in body fat stores (59-62).

Ultimately, it is evident that the pathways involved in T2D pathophysiology are also associated with its risk factors. For instance, vitamin D deficiency is related to poor diet choices and melatonin levels are increased with physical activity (63). Hence, T2D pathogenesis needs to be re-reviewed by exploring these additional pathways in relation to the major risk factors to highlight modifiable pathophysiological defects with lifestyle or therapeutic management. It is also crucial to understand genetic associations with T2D in relation to these pathways and risk factors, aiding in the individualisation of the treatment process.

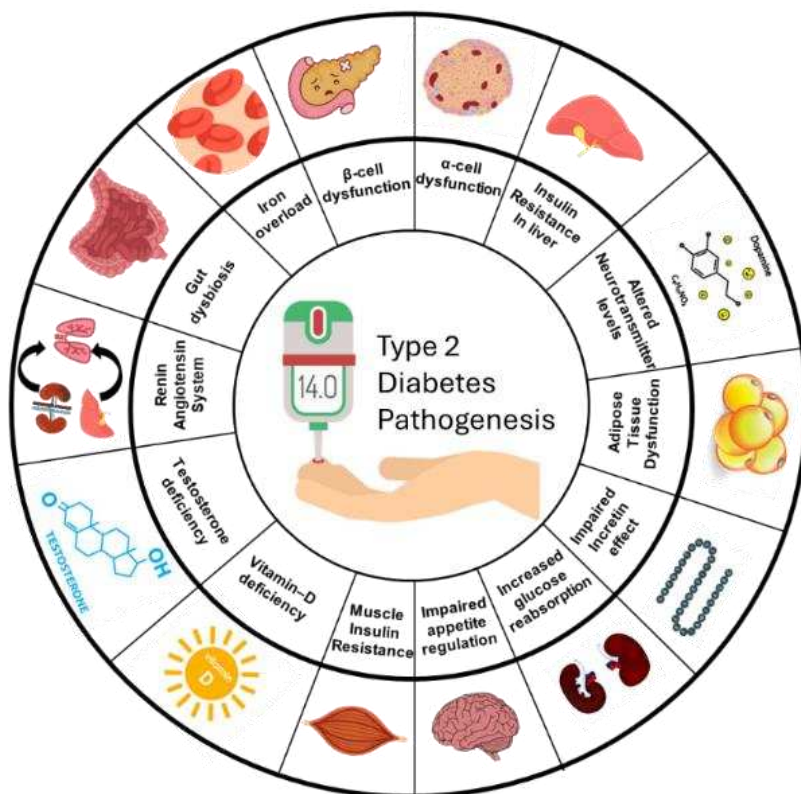


Figure 1.1 The Sweetening Sixteen Hallmarks of Type 2 Diabetes

1.2 Pancreatic regulation of glucose homeostasis

1.2.1 The pancreas anatomy and function

The pancreas is composed of two distinct parts, each serving a different function (Figure 1.2). The exocrine pancreas, which consists of acinar cells, secretes pancreatic juice containing digestive enzymes such as amylase, pancreatic lipase, and trypsinogen (64). This juice is released through a system of pancreatic ducts into the duodenum to aid in the digestion of proteins, fats, and carbohydrates following a meal (65).

On the other hand, the endocrine pancreas releases pancreatic hormones, such as insulin and glucagon, into the bloodstream to regulate glucose homeostasis. Endocrine cells are clustered into small island-like structures scattered within exocrine pancreatic tissue (66). These structures were discovered by Paul Langerhans in 1869 and were subsequently dubbed the islets of Langerhans (Figure 1.2) (67).

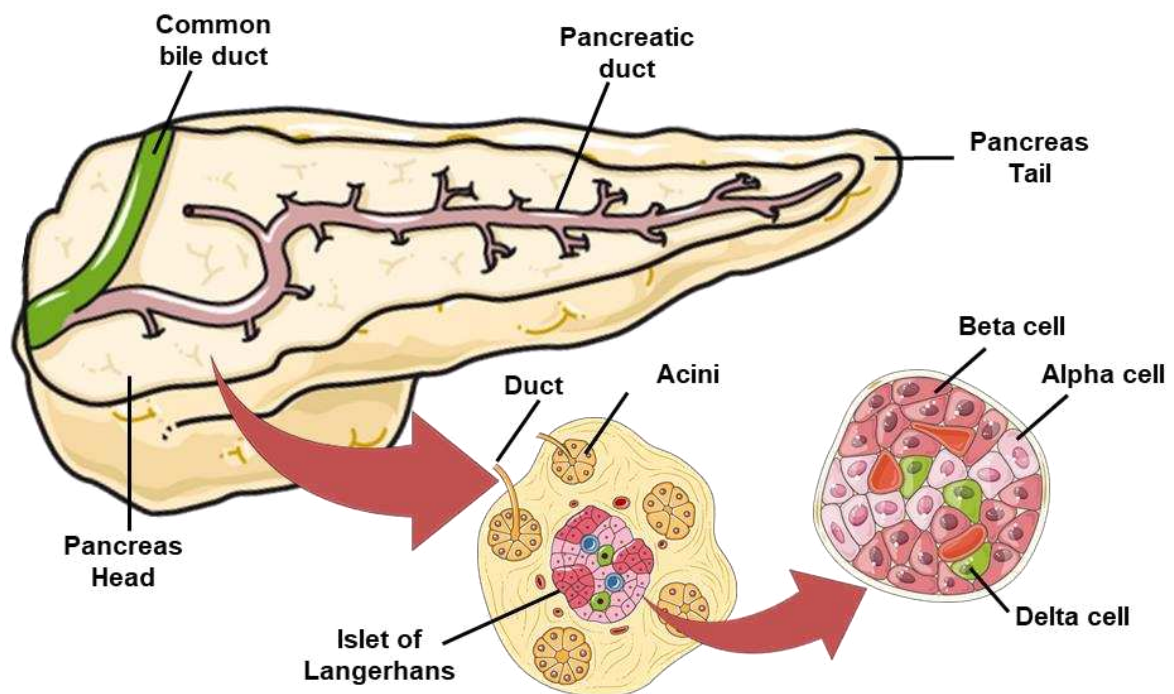


Figure 1.2 Key anatomical Features of the Human Pancreas

1.2.2 The islets of Langerhans and pancreatic hormones

The islets of Langerhans account for 1-2% of the entire pancreas, with a total estimate of between 3.2-14.8 million islets in a human pancreas (68-70) and the highest density in the tail region (71). Pancreatic islets are comprised of five different cell types, 65-80% β -cells (insulin, amylin, and C-peptide-producing), 15-20% α -cells (glucagon-producing), 3-10% δ -cells

(somatostatin-producing), 3-5% γ -cells (pancreatic polypeptide-producing), and ϵ -cells (ghrelin-producing) (72-75). While the cellular composition of pancreatic islets is similar across different species, the cytoarchitecture varies. In human islets, the various endocrine cells are randomly distributed throughout the islet (74). However, in rodents, islet architecture is different and more organised, with the core consisting of β -cells and other endocrine cells such as α -cells in the periphery (76, 77).

Despite secreting different hormones, the different cell types in the islet communicate with each other to function as an integrated unit to regulate glucose homeostasis (78). The main two cells that regulate glucose levels in the bloodstream are insulin-secreting β -cells and glucagon-secreting α -cells, which have opposing effects and secrete their hormones during different feeding states (79, 80). Insulin release is stimulated after a meal, by high blood glucose levels; insulin travels in the bloodstream to peripheral tissues such as muscle, liver, and adipose tissue where it docks to its receptor, enabling glucose uptake and subsequently reducing blood glucose levels (81). It promotes anabolic processes such as glycogenesis (82), lipogenesis (83) and the incorporation of amino acids into proteins (84).

On the other hand, glucagon is secreted in response to low exogenous glucose levels, during the fasting state in sleep or between meals (85-87). Glucagon drives counter-regulatory catabolic processes such as hepatic gluconeogenesis and glycogenolysis, to increase endogenous blood glucose levels (88, 89). While insulin and glucagon play a primary role in glucose regulation, it is important to consider the additional hormones secreted by islet-derived cells, such as somatostatin, in maintaining glucose homeostasis.

The δ -cells secrete somatostatin, which functions importantly through paracrine hormone signalling to potently suppress both insulin and glucagon secretion. Pancreatic polypeptide

(PP), secreted by γ -cells, also plays a role in regulating the secretion of endocrine hormones as well as exocrine pancreas function. Its main function is to inhibit gastric emptying and biliary secretion, modulating food digestion in the intestine. Lastly, ϵ -cells secrete ghrelin, the hunger hormone, which regulates various β -cell functions, including inhibition of insulin secretion and increasing cellular growth by inhibiting β -cell apoptosis. The number of ϵ -cells changes during development; it is highest during foetal development and is significantly reduced in adults (90). This is believed to be due to their ability to act as progenitors, giving rise to PP or ϵ -cells. Therefore, regulation of glucose homeostasis is a complex process that involves the interplay of multiple hormones and signalling pathways.

1.2.3 Understanding insulin: synthesis and signalling

Insulin biosynthesis

Insulin is a 51-residue anabolic protein secreted by β -cells in the pancreatic islets of Langerhans. It was first discovered as an anti-diabetic hormone by Frederick Banting and Charles Best in 1921, building on an earlier hypothesis by Sir Edward Albert Sharpey-Schafer, who first postulated its existence in 1916 (91, 92). Banting and Best isolated insulin from pancreatic extracts, demonstrating its efficacy in lowering blood glucose levels, revolutionizing diabetes management. It was also the first peptide hormone to be purified and crystallized by Abel in 1926, aiding in its clinical application (92, 93).

Insulin biosynthesis is a vital process in the body as it is essential for maintaining homeostasis. The human insulin gene (*Ins*), localised in the short arm of chromosome 11 and composed of three exons and two introns, plays a significant role in insulin biosynthesis (94, 95). The transcription of the *Ins* gene is tightly regulated in pancreatic β -cells, whereby alterations in

ambient nutrient levels such as increased glucose levels promote increased insulin gene transcription (96). Additionally, several transcription factors, including PDX-1, NeuroD1/BETA2, and MAFA, control insulin gene transcription by binding to its promoter region (96). Mutations in the *Ins* gene as well as these transcription factors can lead to dysfunctional insulin biosynthesis and post translational processing, contributing to diabetes mellitus. In fact, most mutations in the insulin gene are associated with impaired proinsulin folding leading to progressive ER stress, β -cell death, and subsequently neonatal diabetes (97, 98).

Despite the elucidation of the insulin amino acid sequence and its subsequent two chain heterodimer tertiary structure in the mid-1950s, the mechanisms of insulin biosynthesis were not resolved until 10 years later when the proinsulin and preproinsulin precursors were discovered (99-101). Preproinsulin, a 12 kDa single-chain polypeptide precursor consisting of proinsulin and a 24-residue hydrophobic signal sequence, is translated from the insulin gene in the rough endoplasmic reticulum (rER) in response to increased glucose levels (102). Preproinsulin is then translocated into the lumen of the rER where the signal peptide is cleaved by a signal peptidase into proinsulin (102). Proinsulin is a 9 kDa polypeptide precursor, consisting of an A chain (21 amino acids) and a B chain (30 amino acids) joined by the intervening C domain segment. The C domain consists of 30-35 residues flanked by dibasic residues which can be cleaved to yield mature insulin and a C-peptide fragment. In the cisternae of the rER, proinsulin undergoes further processing by rapid folding and three cysteine disulfide bond formation into a native tertiary precursor structure (103). The folded proinsulin is then sorted and packaged into early secretory granules along with the proprotein convertases PC2, PC1/3 and carboxypeptidase E in the trans-Golgi network (TGN) (104). The

cleavage products are then stored in maturing secretory granules, along with other less abundant β -cell secretory products and islet amyloid polypeptide (IAPP or amylin), until secretion (102, 104-106). This process is depicted in Figure 1.3.

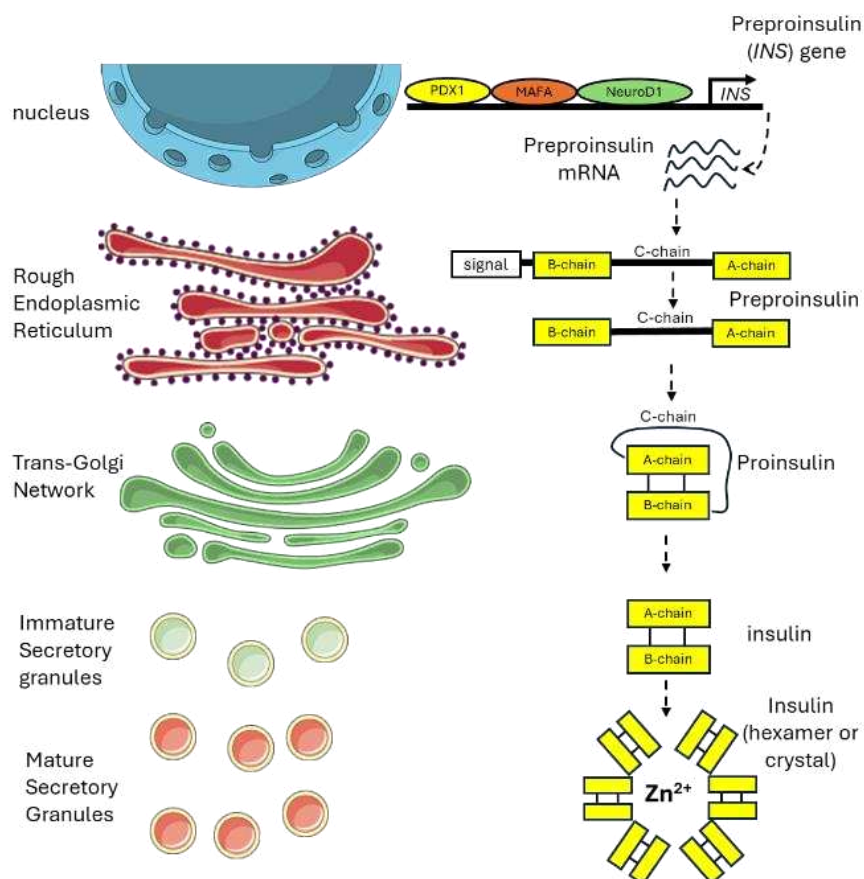


Figure 1.3 Insulin Biosynthesis Pathways

Insulin structure

The 3D structure of insulin has been studied extensively with X-ray crystallography and NMR spectroscopy. Currently there are three main conformational forms of insulin identified: hexamers, zinc-free dimers (T₂), and monomeric fragments (107). The conformational form adopted by insulin is dependent on its concentration, surrounding pH and presence of divalent metal ions (102). At low concentrations (~ 0.001 mM) in the circulation, insulin exists

primarily as a monomeric active form, that is capable of insulin receptor (IR) binding. As the insulin concentration rises and a favourable neutral pH is reached (pH~6.0), insulin monomers readily self-aggregate to form dimers (102). In the dense core of β -cell secretory granules, where insulin concentration is high (40 mM), insulin dimers undergo micro-crystallisation in the presence of Zn^{2+} ions into insoluble hexamer crystalline complexes (102, 107). The hexameric 2- Zn^{2+} insulin (T6), discovered by Dorothy C. Hodgkin in 1972, is recognised as the prototypic insulin structure, and is insulin's storage form (108). This 35 kDa structure is comprised of 6 insulin molecules arranged as three dimers coordinated by two axial Zn^{2+} ions within the central axis of the hexamer (102, 107). Upon secretion into the circulation down the insulin concentration gradient, insulin hexamers dissociate into monomers with the aid of electrostatic repulsion (106, 107).

Insulin signal transduction

Insulin mediates its effects by binding and activating the IR, a transmembrane receptor belonging to the large receptor tyrosine kinase superfamily activated by insulin as well as IGF-I, and IGF-II (109). Metabolically, IR activation promotes a host of cellular signalling pathways regulating glucose uptake into peripheral tissues, protein and fat synthesis, RNA and DNA synthesis, cell growth and differentiation (109, 110).

Insulin signal transduction is triggered by insulin binding to the alpha subunits of the IR, inducing a conformational change that activates the intrinsic tyrosine kinase activity beta subunits (109). This results in the trans-phosphorylation among beta subunits, furthering activating the kinase and creating pleckstrin homology (PH) and phospho-tyrosine binding (PTB) sites to recruit receptor substrates (109). The best characterised receptor substrates are members of the insulin receptor substrate (IRS) family IRS1-6 and the adaptor Src, which act

as scaffolds to organise and mediate downstream signalling complexes (111, 112). Receptor substrates within the IR/IRS complex are phosphorylated on multiple tyrosine residues revealing binding sites for Src homology 2 (SH2) domain proteins, recruiting them in the vicinity of the receptor (112-114). Subsequent activation of these proteins initiates a series of signalling pathways mediating insulin actions, the most crucial of which are the phosphatidylinositol 3-kinase (PI3K)/AKT pathway and the Raf/Ras/MEK/MAPK (Mitogen activated protein kinase) pathway (109, 110). Whilst the PI3K pathway exclusively emanates from the IRS node and is responsible for most metabolic effects of insulin, the MAPK pathway is connected to both the IRS and the Shc pathways and is involved in the regulation of gene expression, and, in cooperation with the PI3K pathway, induction of β -cell growth and differentiation (109, 110).

The main metabolic effect of insulin is preventing postprandial hyperglycaemia by stimulating glucose transport in peripheral tissues, mainly adipose tissue, and skeletal and cardiac muscle. This is accomplished by exocytosis and translocation of the insulin-sensitive glucose transporter GLUT4, one of the 13 human glucose transporters, from intracellular storage vesicles (GSVs) to the plasma membrane (110). Although the exact mechanism behind this process is far from completely understood, it is known that the PI3K/PDK1/AKT2 pathway is involved in translocation of GSVs through phosphorylation of the GTPase-activating protein AS160 which in turn activates small G proteins called RAB which are involved in membrane trafficking (110). Additional studies also suggest that glucose transport via GLUT4 is also mediated by exercise-induced AMPK-dependent mechanisms. GLUT4 is highly expressed in adipose tissue and skeletal muscle and facilitates hexose transport across membranes via an ATP-independent facilitative diffusion mechanism (110). Overall, an impaired ability of

insulin to bind and activate IR, and thus induce GLUT4 translocation from intracellular GSVs, contributes to postprandial hyperglycaemia and insulin resistance.

1.2.4 Glucose-stimulated insulin secretion (GSIS)

Endocrine cells within the islets of Langerhans release their respective hormones in response to external signals such as nutrient intake or stress. Pathways underlying the mechanisms that translate the stimulus into hormone release are called stimulus secretion coupling or stimulus dependent exocytosis; in the case of insulin release from β -cells in response to high blood glucose levels, this is referred to as glucose-stimulated insulin secretion (GSIS) (115). GSIS is a complex process involving multiple steps, including β -cell nutrient sensing, metabolism and subsequently insulin exocytosis (Figure 1.4). The main nutrient sensed by β -cells is elevated postprandial glucose levels, triggering insulin secretion (116). GSIS begins with the uptake of glucose from the circulation via the facilitative glucose transporter GLUT2 (SLC2A2), located on the surface of β -cells (115).

Once inside the cell, high glucose concentrations induce increased glucose metabolism, the rate of which within β -cells determines the magnitude of the insulin secretion response. Glucose metabolism involves several biochemical pathways including glycolysis, the tricarboxylic acid (TCA) cycle, and oxidative phosphorylation (OXPHOS). Upon entry to pancreatic β -cells via the GLUT2 transporter, intracellular glucose is rapidly phosphorylated by glucokinase to glucose-6-phosphate, initiating glycolysis (117-119). In the cytosol, glycolysis mainly metabolizes glucose to pyruvate, simultaneously generating ATP and NADH (120). In β -cells, pyruvate primarily fuels mitochondrial metabolism via two equal pathways: conversion into acetyl-CoA by the pyruvate dehydrogenase (PDH) complex for

entry into the mitochondrial TCA cycle, and anaplerotic conversion into oxaloacetate by pyruvate carboxylase (PC) (120-122). This dual pathway ensures optimal functioning of the TCA cycle, crucial for robust mitochondrial metabolism and sustained ATP generation. Additionally, β -cells possess robust NADH shuttle systems, including glycerol phosphate and malate-aspartate shuttles, import NADH and malate into the mitochondria, converting them into FADH_2 and NADH, respectively (123-125). These shuttles provide critical reducing equivalents that feed directly into the mitochondrial respiratory chain, fuelling OXPHOS, and thereby increasing adenosine triphosphate (ATP) synthesis (124). These combined processes substantially elevate the ATP/ADP ratio essential for insulin secretion.

In addition to this canonical mitochondrial ATP synthesis, β -cells further amplify insulin secretion through mitochondrial-cytosolic pyruvate cycling pathways, collectively known as the phosphoenolpyruvate (PEP) cycle (126, 127). Pyruvate or TCA cycle intermediates such as oxaloacetate generated within mitochondria are converted to PEP by mitochondrial PEP carboxykinase (PEPCK-M) (127). Cytosolic PEP is subsequently converted to pyruvate via pyruvate kinase, generating additional cytosolic PEP. Furthermore, cytosolic PEP can re-enter mitochondria via oxaloacetate and malate intermediates, thus replenishing mitochondrial TCA cycle intermediates (anaplerosis), and sustaining mitochondrial energy production capacity (128). The PEP cycle not only amplifies cytosolic ATP production but also provides an anaplerosis function, reinforcing mitochondrial metabolic efficiency essential for sustained insulin secretion (129). This cumulative elevation in the ATP/ADP ratio arising from both mitochondrial OXPHOS and cytosolic ATP generation then triggers the closure of ATP-sensitive K^+ channels (KATP -channels), depolarization of the cell membrane, followed by opening of the voltage-dependent Ca^{2+} channels (VDCCs) (130). The opening of the VDCCs

allows calcium ion influx into the cell, triggering the fusion of insulin-containing dense-core secretory vesicles with the cell membrane, and exocytosis of readily available insulin hexamers into the circulation (130).

Key molecules are required to mediate fusion of dense-core secretory vesicles containing insulin with the cell membrane, these are members of the superfamily of the soluble N-ethylmaleimide-sensitive factor attachment protein (SNAP) receptor proteins (SNAREs), mainly synaptosomal-associated protein of 25 kDa (SNAP-25), syntaxin-1 and synaptobrevin 2 (or vesicle-associated membrane protein VAMP2) as well as Sec1/Munc18-like (SM) proteins, together forming the SNARE complex (131-133). Fusion is initiated when synaptobrevin 2 (v-SNARE), embedded in the vesicle membrane, fuses with syntaxin-1 and SNAP-25 (t-SNAREs), located in the target cell membrane, and Munc-18 plays a key regulatory role (131-133). Moreover, calcium sensing proteins, known as synaptotagmins, which are highly expressed in neurons and endocrine cells, form a complex with the SNAREs facilitating and initiating the vesicle-cell membrane fusion process (134, 135).

This insulin secretion process is biphasic, with the first phase peaking around five minutes post glucose stimulation, releasing most of the insulin, followed by a second slower phase secreting the remainder of the insulin (136-139). This process is also tightly regulated to ensure that insulin is released in response to increased blood glucose levels, thereby maintaining glucose homeostasis.

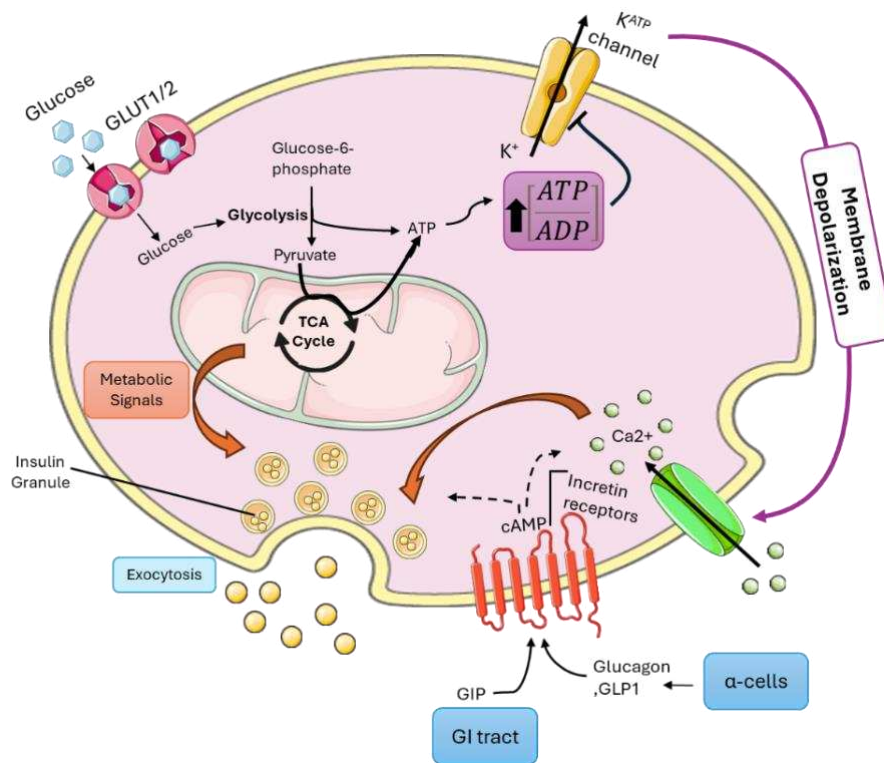


Figure 1.4 Mechanisms Controlling Pancreatic β -Cell Glucose Stimulated Insulin Secretion

1.2.5 Modulation of pancreatic hormone secretion by key external factors

GSIS is a tightly regulated process, whereby its initiation, potentiation and inhibition are mediated by a variety of modulators, primarily G protein-coupled receptors (GPCRs) (115). Glucose not only triggers insulin secretion, but also amplifies it through activation of metabolism cyclic adenosine-monophosphate (cAMP) pathway or via the incretin hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulintropic peptide (GIP) (130). The glucose-stimulated increase in intracellular glucose metabolism and ATP production also results in increased cAMP production by adenylate cyclase (AC) (140), and subsequently cAMP associated signalling pathways like protein kinase (PKA) and a cAMP regulated guanine nucleotide exchange factor known as exchange protein directly activated by cAMP 2

(Epac2) (141) are activated. Epac2 triggers the release of calcium from intracellular vesicles, which increases Ca^{2+} influx and controls granule density near the cell membrane (140). Meanwhile, PKA operates by phosphorylating KATP and calcium channels, increasing the number of calcium-sensitive insulin-containing secretory granules, and the likelihood of secretory vesicle release, respectively. Both processes result in the amplification of insulin secretion in different ways converging on the outcome of insulin secretion amplification (115). The incretin hormones, GLP-1 and GIP, secreted from intestinal cells in response to glucose, fructose, amino acid and free fatty acid (FFA) ingestion (141), also amplify insulin secretion by up to 50% via the incretin effect (142, 143). The action of these incretins is also mediated by cAMP accumulation and activation of downstream effectors of the cAMP pathway by binding to their corresponding receptors and initiating $\text{G}\alpha_s$ coupling (144, 145). Moreover, the incretins can also potentiate insulin secretion by acting directly on calcium stores and VDCCs to increase calcium influx, as well as inducing PKA-mediated phosphorylation and activation of proteins involved in vesicle-membrane fusion (146, 147).

1.3 The incretin hormones: physiological effects in glucose metabolism

1.3.1 The incretin effect

The incretin effect refers to the phenomenon where oral glucose intake upon meal ingestion, triggers a two- to three-fold higher insulin secretion response than the same amount of glucose administered intravenously (148, 149). In fact, this effect is responsible for up to 70% of the insulin response to oral glucose intake and is thus a major contributor to glucose

tolerance (150). Gastrointestinal Mediated Glucose Disposal (GIGD), or “the poor man’s incretin estimate”, is a physiological mechanism in which glucose is disposed of or taken up in response to nutrient intake. After meal ingestion, the gastrointestinal tract signals and activates mechanisms that enable disposal and uptake of 2/3 of the glucose load from the bloodstream into peripheral tissues, such as muscle, liver and adipose tissues for energy production and storage, on top of those activated by increases in plasma glucose alone (151). Moreover, the incretin effect is dose-dependent, meaning that the insulin secretion increases progressively and proportionately with the amount of glucose ingested, eliciting identical plasma glucose concentrations (150). This finely tuned response helps ensure that blood glucose levels are effectively controlled regardless of the amount of carbohydrate ingested, preventing both hyperglycaemia (too high blood glucose) and hypoglycaemia (too low blood glucose). Consequently, the incretin effect is one of the most important determinants of postprandial glucose excursions.

The incretin effect and GIGD are mediated by the secretion of incretin hormones, glucagon-like peptide-1 (GLP-1) and GIP (124). These gut peptides are released from the intestinal endocrine L and K cells, respectively, upon nutrient intake. These hormones slow down the rate of gastric emptying, reduce appetite and body weight, and enhance insulin secretion in a glucose-dependent manner (151). This helps minimise the risk of hypoglycaemia while playing a vital role in regulating blood glucose levels.

The incretin effect plays a crucial role in glucose tolerance and postprandial glucose excursions. As expected, this effect is often reduced or absent in patients with T2D, resulting in postprandial hyperglycaemia due to a near-total loss of GIGD (152). While studies on GIP concentrations have been inconsistent due to low power, confounding factors, or technical

limitations, several studies have reported a significant impairment in postprandial GLP-1 response and secretion in T2D patients (153, 154). This impairment is thought to be secondary to the disease, and factors such as the severity of T2D, increased BMI, obesity, and insulin resistance have been linked to this defect (155). Therefore, understanding and harnessing the incretin effect has become a major focus in the treatment of T2D and obesity.

1.3.2 GLP-1: synthesis, secretion and elimination

GLP-1, or glucagon-like peptide-1, is synthesised in intestinal L cells as a result of the post-translational modification of proglucagon, a protein precursor (Figure 1.5). The proglucagon gene (*Gcg*) encodes the human preproglucagon and parts of the *Gcg* promoter, including four enhancers G1, G2, G3, and G4, as well as a cAMP response element (CRE_{PG}) (156-158). The transcriptional control of *Gcg* is regulated by various mechanisms. These include changes in cAMP levels, exposure to amino acids, or association with specific homeodomain protein transcription factors (159-161). For example, insulin promotes *Gcg* transcription in intestinal cells via the Wnt signalling pathway but inhibits it in pancreatic α -cells (157, 162, 163). Additionally, individuals with bowel resection or damage show increased *Gcg* expression (164). Just like the varied transcriptional regulation mechanisms, there are distinct patterns of proglucagon (proG) processing after cleavage from preproglucagon. These patterns depend on tissue-specific post-translational modification by prohormone convertases (PC/PCSK), resulting in different proG peptides (165).

The proG spliceosome consists of the bioactive proteins glucagon, GLP-1, GLP-2, oxyntomodulin, glicentin, glicentin-related pancreatic polypeptide (GRPP) and major proglucagon fragment (MPGF) (Figure 1.5) (166). While there is ample literature on glucagon (167-169), GLP-1 (170), GLP-2 (171), and oxyntomodulin (172), the roles of glicentin (173),

GRPP (174), and MPGF are not as well-defined. The abundance and type of prohormone convertase expressed is tissue specific, resulting in differential amounts and forms of proG peptides. In intestinal enteroendocrine L-cells and nucleus tractus solitarius (NTS) neurons, PC1/3 is predominantly expressed. This leads to the release of GLP-1 and GLP-2 from MPGF, with glicentin partially processed to oxyntomodulin and very low levels of glucagon (175-179). ProG processing, and therefore GLP-1 synthesis, is most abundant in the distal small intestine, particularly in the colon.

In contrast, α -cells predominantly express PC2, leading to the complete processing of glicentin to glucagon and the liberation of unprocessed MPGF (180). Studies increasingly show that high glucose levels induce increased PC1/3 expression in α -cells, promoting GLP-1 production from islets via these cells (181, 182). However, despite the expression of PC2 in the brain, only trace amounts of glucagon are found in the CNS (179).

It is well-known that GLP-1 has four main forms processed from proglucagon. These include GLP-1(1-37) and GLP-1(7-37), which result from prohormone cleavage at sites containing two basic amino acids (183, 184). The other two forms, GLP-1(1-36) amide and GLP1(7-36) amide, result from PC cleavage followed by the amidation of the penultimate arginine by a peptidylglycine α -amidating monooxygenase (184, 185).

GLP-1 secretagogues include nutrients such as dietary monosaccharides, lipids, proteins, and certain amino acids, along with neuroendocrine and hormonal factors (186-188). Nutrient-induced stimulation of GLP-1 secretion occurs rapidly through direct contact of L-cells with these nutrients in the distal gut (186). This interaction elicits a rapid rise in plasma GLP-1 levels, occurring 30-60 minutes after ingestion of carbohydrate or proteins, and a delayed, but more sustained response for lipids (189).

Monosaccharides such as glucose, galactose, and fructose, stimulate GLP-1 secretion in a dose dependant manner (188). However, GLP-1 secretion induced by fructose occurs at much lower levels relative to those induced by equivalent glucose loads (190). The uptake of glucose into L-cells is thought to be mediated via GLUT2, SGLT2 and SGLT1, the electrogenic action of which is particularly crucial for coupling the stimulus with GLP-1 secretion at low glucose concentrations (191-194). Upon glucose uptake, the closure of ATP- sensitive KATP channels and the opening of VDCCs leads to membrane depolarisation and calcium influx, respectively (195, 196). Calcium dependent vesicular exocytosis and GLP-1 secretion is then carried out, involving similar cellular machinery to that utilised in GSIS (195, 196).

L-cells detect dietary lipids and proteins through basolaterally localised cell surface receptors. These receptors bind to constituent metabolites after absorption (197). In the case of triglycerides, GPR40 (FFAR1), which is enriched in L-cells, primarily mediates GLP-1 secretion in response to long-chain free fatty acids (198). Meanwhile, GPR119 plays a role in 2-mono-acylglycerol (2-MAG) acting synergistically to induce GLP-1 secretion (199). Similarly to glucose, free fatty acids have also been found to increase GLP-1 secretion through calcium-dependent mechanisms (200).

Protein-induced GLP-1 secretion has been demonstrated and seems to be dose-dependent (201, 202), as evident from a study whereby healthy humans consuming a high-protein diet show greater GLP-1 secretion compared to those on low-protein diets (203). The mechanism driving increased GLP-1 secretion in response to amino acids involves calcium-sensitive L-cell signalling via the Ca^{2+} sensing receptor (CaSR) and peptide transporter 1 (PEPT1) (204), as well as activation of the calcium/calmodulin-dependent kinase II pathway by GPCR6A (205).

Both incretin hormones, GIP and GLP-1, are primarily degraded by the enzyme dipeptidyl peptidase-4 (DPP-4), also known as CD26 (206, 207). This multifunctional serine protease swiftly cleaves endogenous GLP-1, removing a dipeptide from the N-terminal end, and yielding the inactive metabolites GLP-1(9-36) amide and GLP-1(9-37), which are subsequently cleared by the kidneys (208). There are conflicting views on the roles of these metabolites. Some studies suggest that they lack insulinitropic activity and might function as partial antagonists (170, 209, 210), while others show that GLP-1(9-36) amide induces weak insulin secretion, improved glucose handling and improved cardiac output (211-213).

DPP-4 exists as both an integral membrane protein and as a soluble peptidase circulating in blood plasma, upon release of its extracellular domain (214). The membrane-bound form is expressed ubiquitously in various tissues, including intestinal and renal brush borders, vascular endothelium, liver, pancreas, glandular epithelial cells, and immune system cells (215). This ubiquitous two-form expression enables DPP-4 to rapidly shorten the half-life of endogenous GLP-1 to less than two minutes in circulation (216).

The rapid degradation of GLP-1 by DPP-4 has significant implications in the physiological regulation of glucose metabolism and has been a major challenge in the therapeutic use of GLP-1. However, this challenge has been met by the development of GLP-1 analogues that are resistant to DPP-4 cleavage and DPP-4 inhibitors that prevent the degradation of endogenous GLP-1 (214). Both strategies extend the action of GLP-1 and have proven effective in the treatment of T2D.

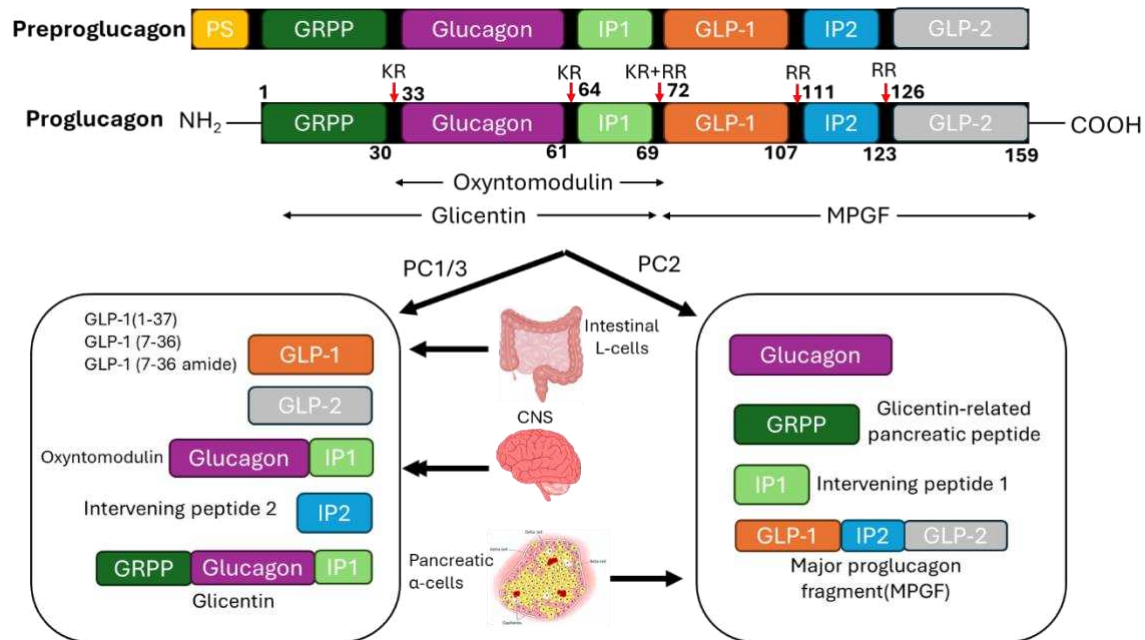


Figure 1.5 Synthesis of Proglucagon and its Derived Peptides such as GLP-1

1.3.3 Neuronal and paracrine regulation of GLP-1 secretion

The observation of biphasic GLP-1 secretion and the distinct localisation of L-cells in the distal versus proximal gut suggest the existence of a neuroendocrine-regulated proximal-to-distal loop. This loop mediates early and delayed GLP-1 responses to nutrient intake. Rapid early GLP-1 secretion can occur through direct interaction of L-cells with nutrients in the proximal gut. However, neuroendocrine factors released in the duodenum can also trigger GLP-1 secretion even before nutrients reach the distal gut. For instance, GIP activates vagal and enteric afferent neurons, which release the neurotransmitters acetylcholine and GRP, stimulating GLP-1 release from the distal gut. Finally, nutrients that reach the distal gut and directly contact L-cells will trigger an additional second phase GLP-1 response (217).

GLP-1 secretion is not confined to intestinal L-cells. Certain glutamatergic neurons in the NTS, which express PC1/3, also secrete GLP-1 in response to various peripheral signals such as leptin (218). This GLP-1 secretion from the central nervous system (CNS) regulates several aspects of feeding behaviour, including food intake and energy balance.

Furthermore, an increasing number of studies highlight the significance of GLP-1 secretion from α -cells for the paracrine regulation of insulin secretion in islets, as opposed to intestinal GLP-1. The activity and expression of PC1/3 in α -cells have been observed to increase under conditions of metabolic stress and high glucose levels (219). Notably, a greater percentage of α -cells from healthy human islets secrete GLP-1 compared to those from mouse islets, indicating its importance in humans (220).

1.3.4 The physiological roles of GLP-1

GLP-1 plays diverse and crucial roles in maintaining physiological functions across several tissues (Figure 1.6). GLP-1 mediates its biological effects by activating the GLP-1 receptor (GLP-1R), a GPCR expressed in several tissues, most commonly the endocrine pancreas, and to a lesser extent, the brain, cardiovascular system, GI tract, immune and reproductive system (221).

A significant amount of research has been conducted on the role of GLP-1 in maintaining glucose homeostasis in the pancreas (221). It does so by stimulating glucose-dependent insulin secretion from β -cells and suppressing glucagon secretion from α -cells. However, the mechanism through which GLP-1 secretion inhibits glucagon secretion remain unclear. Some studies suggest that the inhibitory effect is indirectly mediated through the paracrine action of somatostatin and insulin, both stimulated by GLP-1 and known as potent inhibitors of

glucagon secretion (222, 223). However, this model doesn't explain why GLP-1 induced glucagon inhibition persists in the absence of insulin stimulation or in T1D.

Contrary to the traditional model, there is increasing recognition of a direct model, where α -cell GLP-1R plays a significant role in regulating glucose-dependent glucagon secretion (224, 225). While it was initially thought that α -cells lacked GLP-1R expression due to methodological limitations, recent studies have demonstrated GLP-1R expression using more specific antibodies, fluorescent GLP-1R agonists, and transgenic reporter mice (224, 226-228). Interestingly, recent research reports nutrient-dependent α -cell heterogeneity, where some α -cells secrete GLP-1 in response to high glucose conditions, and others secrete both glucagon and GLP-1 in the presence of amino acids (224, 226). This suggests a bi-directional paracrine and autocrine mechanism, where α -cell-derived GLP-1 signals through the β -cell GLP-1R to potentiate insulin secretion, and through its own GLP-1R to suppress glucagon secretion (229). Indeed, research has found that α -cell-derived GLP-1 is indispensable for glucose homeostasis, compared to intestinal GLP-1 (230). In addition to its insulintropic actions, GLP-1 promotes β -cell proliferation and inhibits apoptosis in human islets, further contributing to glucose homeostasis independent of stimulating insulin secretion (231, 232).

Beyond the pancreas, GLP-1 has physiological effects on other tissues as well. In the gut, it lowers blood glucose levels by slowing gastric emptying and suppressing appetite, potentially aiding in weight loss (233, 234). This effect is thought to occur via the activation of GLP-1R in vagal afferent neurons, particularly the nodose ganglion, leading to the inhibition of stomach peristalsis and an increase in tonic contraction of the pyloric region, thus suppressing gastric motor function (235, 236).

In the brain, GLP-1 influences the CNS to promote satiety, decreasing food intake and potentially further assisting weight management. Accordingly, hypothalamic neuron GLP-1R activation in the arcuate nucleus (ARC), independent of signalling in vagal afferents or activation of GLP-1-producing neurons in the hindbrain, is sufficient for the anorectic effect of peripherally administered GLP-1 analogues (237, 238). Interestingly, native GLP-1 produced in the hindbrain neurons induces a different pattern compared to prolonged GLP-1R agonists (239). Therefore, it remains uncertain whether peripherally or locally derived GLP-1 is more important for regulating satiety and food intake in the brain. GLP-1 also impacts the brain beyond metabolic health. It has been found to affect other neuronal activities, such as synaptic plasticity, memory formation, neuroprotection, and stress responses (240-242).

GLP-1 is increasingly recognised as a key regulator of cardiovascular functions. Clinical research has shown that GLP-1 analogues positively affect heart rate, arrhythmia, and blood pressure in patients with heart failure (243). Moreover, GLP-1 has been shown to elicit cardioprotective effects by improving endothelial function, reducing systemic inflammation, and protecting the heart from ischemic injury (243).

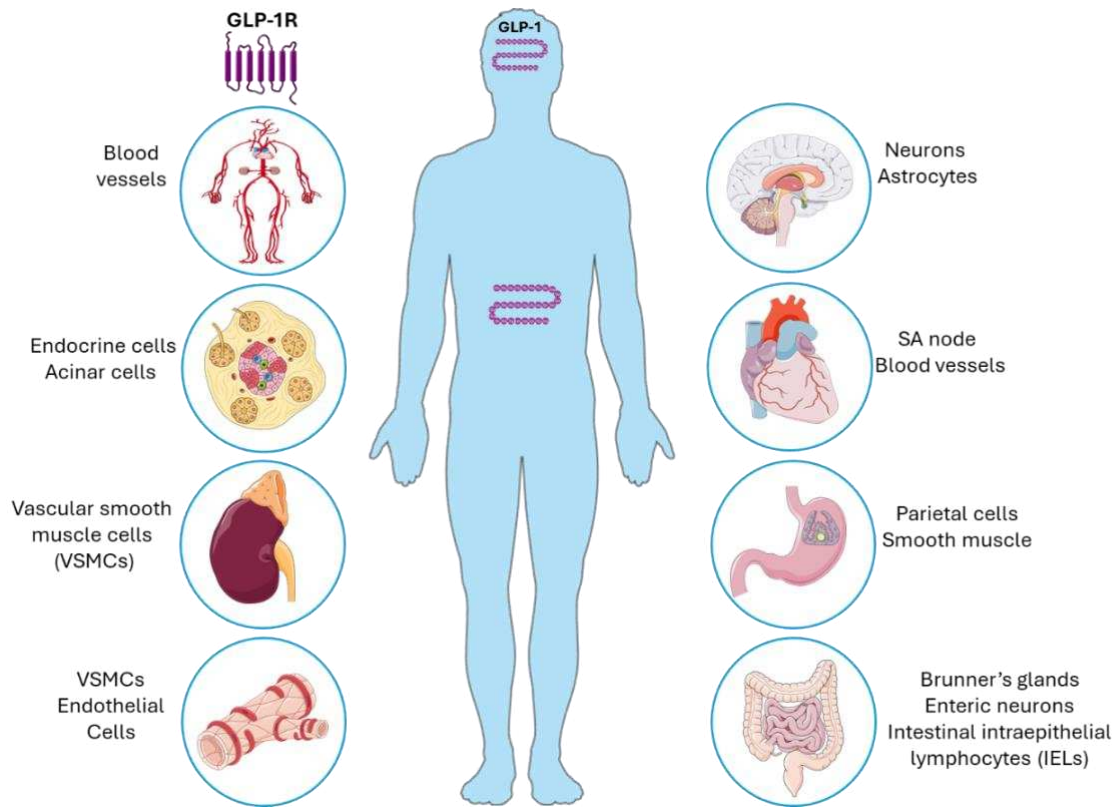


Figure 1 6 The Physiological Roles of GLP-1 and its receptor GLP-1R in Multiple Human Organs

1.3.5 GLP-1 in T2D and obesity

GLP-1 plays a significant role in the management of T2D and obesity, two conditions that have reached epidemic proportions worldwide. It acts as a potent tool against these diseases due to its ability to enhance insulin secretion, suppress glucagon release, slow gastric emptying, and satiate hunger.

The incretin effect is often reduced or absent in patients with T2D and impaired glucose tolerance, which can result in postprandial hyperglycaemia and fasting hyperglucagonemia due the near-total loss of GIGD (244). In particular, previous studies have reported a significant impairment in postprandial GLP-1 responses and secretion in T2D patients (152, 245, 246). Non-surprisingly, these patients often exhibit significantly higher plasma glucose

peaks in response to oral glucose loads compared to healthy control subjects as well as changes in gastric emptying (247). This impairment is thought to be secondary to the disease, and factors such as the severity of T2D, increased BMI, obesity, and insulin resistance have been linked to this defect (248, 249). In fact, glucose tolerance and obesity have also been shown to impair GLP-1 stimulated insulin secretion in an independent additive manner. Here, the incretin effect, particularly the GLP-1 response, was inversely related to glucose tolerance and BMI (249).

Despite, the reduced incretin effect in T2D being much more closely related to the insulinotropic activity of GIP, infusion studies showed that while GIP was almost inactive, GLP-1 was still capable of stimulating insulin secretion in T2D patients (250). These findings prompted the idea of using incretin-based therapies such as GLP-1 analogues and DPP-4 inhibitors to replace GLP-1 levels and manage T2D and obesity.

Yet, it cannot be concluded that T2D progression is characterised by loss of GLP-1 secretion ability until longitudinal studies assessing GLP-1 levels from pre-diabetes to T2D diagnosis stage are done. Also, reduced GLP-1 levels are not a universal characteristic found in all patients (251). As to the ability of incretin-based therapies to rescue insulin secretion, they may be eliciting this through other GLP-1R mediated signalling activities as the concentrations administered are at much higher levels compared to endogenous GLP-1 (251).

Nevertheless, the role of GLP-1 in the early improvement of glycaemic control after Roux-en-Y gastric bypass (RYGB), even before weight loss in obese patients with T2D, hints at its role in the pathophysiology of these diseases. The surgery alters the transit of nutrients in the gut, resulting in significantly heightened postprandial GLP-1 secretion and enhanced β -cell function (252).

1.4 The GLP-1 receptor

1.4.1 G protein-coupled receptors (GPCRs)

The GPCR superfamily consists of the largest group of membrane-bound proteins, with approximately 800 members in vertebrates classified into five classes by the International Union of Basic and Clinical Pharmacology (IUPHAR), based on their evolutionary conservation and sequence similarities. These classes include Rhodopsin (Class A), Secretin (Class B1), Glutamate (Class C), Adhesion (Class B2) and Frizzled and Taste (Class F).

GPCRs are present in various organisms including humans, animals, yeast, and numerous viral genomes. They represent up to 2% of proteins encoded by the human genome. All GPCRs share a similar pattern of structural arrangement, comprising an extracellular N-terminus, a 7 transmembrane (TM) core linked by three intracellular and three extracellular loops, and an intracellular C-terminus. The latter consists of a short helix (H8) running parallel to the membrane and an unstructured tail of varying lengths. Other conserved features include a disulphide bond between ECL1 and ECL2, present in all receptor families. Additionally, several glycosylation sites identified on the extracellular face of various GPCRs play a crucial role in receptor expression and function.

Activation of these GPCRs occurs upon binding with their cognate agonists. This binding triggers a conformational change in the GPCR, enabling it to recruit G proteins or other primary transducers, such as GPCR kinases (GRKs) and β -arrestin at the intracellular face of the receptor. This process initiates the activation of downstream signalling pathways via secondary messengers such as cAMP and inositol phosphates, leading ultimately to physiological responses in cells.

These receptors are vital for regulating numerous physiological mechanisms. They act as mediators of signal transduction, converting external stimuli such as peptides, light photons, biological amines, nucleotides, lipids, and ions into intracellular signals within cells. Almost one-third of FDA-approved therapeutics are GPCR targeting, with drugs developed or in development for treating a wide range of disease conditions, including schizophrenia, pain, hypertension, and diabetes, among others.

1.4.2 Class B1/secretin family of GPCRs

The class B1/secretin GPCRs comprise a subfamily of 15 receptors characterised by their large extracellular N-terminus domains, which are essential for high-affinity ligand binding (253). The class B1 GPCRs include receptors that respond to long peptide hormones such as the secretin receptor (SECR), the glucagon receptor (GCGR), and the glucagon like-peptide 1 and 2 receptors (GLP-1R and GLP-2R), the gastric inhibitory polypeptide receptor (GIPR), pituitary adenylate cyclase-activating peptide receptor (PAC1), vasoactive intestinal peptide receptors (VIP1 and 2), parathyroid hormone receptors (PTH1R and 2), amylin receptor (AMYR), and calcitonin receptor (CALCR) (254). These receptors and their corresponding peptide hormones are therapeutic targets for several human diseases, such as diabetes (GCGR, GLP-1R, AMYR and GIPR) (255-259), osteoporosis (CALCR, PTH1R) (260, 261), neurodegeneration (VIP1R, PAC1, GLP-1R) (262, 263), hypoglycaemia (GCGR) (264), short bowel syndrome (GLP-2R) (265) and cardiovascular disease (GLP-1R) (266). Beyond approved therapeutics, these receptors are also implicated in the pathophysiology of post-traumatic stress disorder (263), inflammation (267), nociception (268), and stress responses (269).

Human class B1 GPCRs exhibit masses in the range of ~ 47-66 kDa and a length of ~470 residues (270). As of 2022, structures for all class B1 receptors coupled to heterotrimeric $G\alpha_s$ -proteins have been resolved using cryo-electron microscopy (cryo-EM) and X-ray crystallography, with findings deposited in the Protein Data Bank (PDB) (270, 271). These structures have enhanced our understanding of class B1 receptor ligand binding and provided a solid platform for structure-based drug design. Additionally, the receptor and peptide dynamics from these structures have revealed key insights into their activation mechanisms. The Secretin receptor family, which is structurally similar, is characterised by a large extracellular N-terminal domain (approximately 120 residues), seven transmembrane domains (TMDs), separated by three intracellular loops (ICL1-3), three extracellular loops (ECL1-3), and a relatively short intracellular C-terminal domain (254, 270). The N-terminal extracellular domain (ECD) of the class B1 family serves as the primary receptor-ligand interaction site and is crucial for high affinity binding to the C-terminal end of the ligand. It has a distinct structure that includes two antiparallel β -sheets ($\beta 1$, $\beta 2$, $\beta 3$, and $\beta 4$), an N-terminal α -helix, and three disulphide bridges formed from six cysteines (272, 273). Additionally, a conserved disulphide bridge exists between cysteine residues in ECL1 and ECL2 (272). The peptide hormone ligands, like their corresponding receptors, have 27-44 amino acid residues and adopt an α -helical conformation upon binding to their respective receptors (274).

There are two proposed mechanisms for the activation of class B1 GPCRs. The first, known as "the two-step model," involves activating the receptor in two stages. The C-terminal portion of the ligand first binds to the N-terminal ECD of the receptor with high affinity and specificity (275). Then, the N-terminal end of the ligand binds to the core domain of the receptor, leading to a conformational change in the receptor, subsequent receptor activation, and G protein

recruitment (275, 276). The second mechanism, known as "the endogenous agonist" hypothesis, proposes that a hidden WDN sequence motif is exposed upon ligand-induced conformational change in the receptor N-terminal domain (277, 278). This motif acts as an agonist ligand, interacting with the receptor core to activate it (277, 278).

Observations of active-state class B1 GPCR structures reveal common conformational changes upon receptor activation amongst family members. These changes include an outward movement at the top of transmembrane helix 6 (TM6) / ECL3 / TM7 and an inward movement of TM1, forming a 'V-shaped' pocket that accommodates the peptide ligand on the extracellular side (279). At the intracellular side, a sharp kink in the middle of the P^{6.47}xxG^{6.50} motif leads to TM6 outward movement, opening the receptor base to allow G protein recruitment (280). The $\alpha 5$ helix of G α_s forms a highly conserved interaction with TM2, 3, and ICL2 (281). Additionally, the HETx motif (H^{2.50}E^{3.50}T^{6.42}x^{7.57}), adjacent to the cytoplasmic side of the receptor, also plays a crucial role in receptor inactivation and G protein recruitment (282, 283). Thus, G α_s -protein coupling requires disruption of HETx polar interactions, which is observed for all G α_s -protein bound class B1 receptors, indicating a common G α_s recruitment mechanism (283). On the other hand, the receptor ECD, ECL1, and TM6 / ECL3 / TM7 conformations vary amongst class B1 GPCRs (279, 284-286). The ECD region is poorly resolved in all cryo-EM maps, reflecting greater flexibility/mobility in the region, further validated by molecular dynamics (MD) simulations (279). Furthermore, the ECD conformation of the receptors is agonist-dependent, as indicated by the different ECD orientations in cryo-EM structures of GLP-1R bound to peptide, non-peptide, and allosteric ligands (287). Collectively, the ECD and ECLs of class B1 GPCRs demonstrate a structural capacity to regulate the binding of different peptides.

1.4.3 GLP-1R structure, binding and activation

Structure

The GLP-1R is a classic class B1 GPCR, that binds to the GLP-1 peptide hormone mediating its physiological effects in pancreatic β -cells, cardiovascular system, and the brain. Since the GLP-1R is a key pharmaceutical target for T2D and obesity, understanding its structure, binding, and activation mechanisms is crucial.

Thorens *et al.* first cloned the cDNA for the GLP-1R in 1992 from a rat pancreatic islet cDNA library in COS cells (288), and the human receptor was cloned the following year (289, 290). The cloning revealed that the receptor consists of 463 residues, sharing sequence homology with the secretin (SCTR), calcitonin (CALCR), and parathyroid hormone (PTHrP) receptors (289, 290). In addition, the structure of the GLP-1R also resembles other class B1 receptor structures, featuring seven TMDs, a large amino-terminal hydrophobic region, and a hydrophilic domain of 120 amino acids (291). This domain contains three N-linked glycosylation sites, essential for standard receptor function, folding, and trafficking to the cell surface (292).

Obtaining high-resolution GLP-1R structures through X-ray crystallography is challenging, hence, single particle cryo-EM has become a favoured method for revealing high-resolution signalling complexes. These complexes include the activated GLP-1R with peptide agonists, non-peptide agonists, antagonists, allosteric modulators, heterotrimeric G proteins, and auxiliary molecules like cholesterol (293). To date, 43 different GLP-1R structures, either complexed or not with various agonists, have been determined using cryo-EM and X-ray crystallography (293, 294) (Table 1.1).

Table 1.1 List of all available GLP-1R PDB structures. Details include bound agonists, state, auxiliary molecules, structure length, and references.

<i>PDB ID</i>	Ligand	Method	Receptor Length	State	Signalling Proteins	Auxiliary Molecules	Date	Reference
3C59	Exendin-4	X-ray	ECD	Active	None	10M	02/08	Reedtz-Rung, S. (2008)
3IOL	Glucagon	X-ray	ECD	Active	None	10M	10/09	Reedtz-Rung, S. (2010)
3C5T	Exendin-4	X-ray	ECD	Active	None	10M	10/09	Reedtz-Rung, S. (2010)
4ZGM	Semaglutide backbone	X-ray	ECD	Active	None	8AIB, 34R-GLP-1(7-37)-OH, 32M	09/15	Reedtz-Rung, S. (2015)
5E94	None	X-ray	ECD	Inactive	None	Fab7F38 light chain, Fab7F38 heavy chain	08/16	Soroka, V. et al 2016
5VAI	GLP-1	Cryo-EM	Full	Active	G α s, G β 1, G γ 2	Nb35	05/17	Zhang et al. 2017
5NX2	Truncated peptide agonist	X-ray	Full	Intermediate	None	Chitin/ NAG, SOG	05/17	Jazayeri et al., 2017
5VEX	NNC0640 (Negative allosteric modulator)/97V	X-ray	Full	Inactive	None	T4-Lysozyme, Endolysin Chimera	05/17	Song et al., 2017
5VEW	PF-06372222	X-ray	Full	Inactive	None	Endolysin Chimera, oleic acid (OLA), OLC, T4-Lysozyme	05/17	Song et al., 2017
6GB1	Peptide 11 (exendin-4 derived GLP-1 and GCG dual agonist)	X-ray	ECD	Active	None	Sulfate ion, AIB, HEZ(hexane-1,6-diol)	06/18	Schreuder and Liesum, 2018
50TX	GLP-1 variant Ala8Cys/Thr11Cys	X-ray	ECD	Active	None	None	07/18	Oddo et al., 2018
50TV	GLP-1 variant AlaCys/Thr11Hcs	X-ray	ECD	Active	None	None	07/18	Oddo et al., 2018
50TW	GLP-1 variant Ala8Hcs/Thr11Cys	X-ray	ECD	Active	None	None	07/18	Oddo et al., 2018
50TT	Exendin-4 variant Gly2Hcs/Thr5Hcs	X-ray	ECD	Active	None	None	07/18	Oddo et al., 2018
50TU	GLP-1 variant Ala8Hcs/Thr11Hcs	X-ray	ECD	Active	None	None	07/18	Oddo et al., 2018
6B3J	Exendin-P5	Cryo-EM	Full	Active	G α s, G β 1, G γ 2	Nb35	08/18	Liang et al., 2018
6KJV	PF-06372222	X-ray	TM domain,	Inactive	None	T4-Lysozyme, Endolysin Chimera OLC	11/19	Xu et al., 2019

			Thermal-stabilised M9					
6KK1	PF-06372222	X-ray	TM domain, Thermal-stabilised M8	Inactive	None	T4-Lysozyme, Endolysin Chimera	11/19	Xu et al., 2019
6KK7	PF-06372222	X-ray	TM domain, Thermal-stabilised M6	Inactive	None	T4-Lysozyme, Endolysin Chimera	11/19	Xu et al., 2019
6ORV	TT-OAD2(non-peptide agonist)/N2V	Cryo-EM	Full	Active	Gαs, Gβ1, Gy2	Nb35	1/20	Zhao et al. 2020
6LN2	PF-06372222 (Negative allosteric modulator)/97Y	X-ray	Full	Inactive	None	Fab7F38 light chain Fab7F38 heavy chain Zinc ion, NAG	03/20	Wu et al., 2020
6VCB	GLP-1 and LSN3160440/QW 7 (positive allosteric modulator)	Cryo-EM	Full	Active	Gαs, Gβ1, Gy2	Nb35	07/20	Bueno et al, 2020
7C2E	RGT1383 (small molecule full agonist)	Cryo-EM	Full	Active	Gαs, Gβ1, Gy2	Nb35	08/20	Ma et al. 2020
6X18	GLP-1	Cryo-EM	Full	Active	Gαs, Gβ1, Gy2	Nb35	09/20	Zhang et al. 2020
6X1A	Danuglipron	Cryo-EM	Full	Active	Gαs, Gβ1, Gy2	Nb35	09/20	Zhang et al. 2020
6X19	CHU-128 (non-peptide agonist)	Cryo-EM	Full	Active	Gαs, Gβ1, Gy2	Nb35	09/20	Zhang et al. 2020
6XOX	LY3502970 or V6G	Cryo-EM	Full	Active	Gαs, Gβ1, Gy2	Nb35, scFv16	11/20	Kawai et al, 2020
7LCI	Danuglipron(PF-06882961) small molecule agonist	Cryo-EM	Full	Active	Gαs, Gβ1, Gy2	UK4	01/21	Zhang et al. 2021
7LCJ	Danuglipron	Cryo-EM	Full	Active	None	UK4	01/21	Zhang et al. 2021
7LCK	Danuglipron	Cryo-EM	Full	Active	None	UK4	01/21	Zhang et al. 2021
7KIO	Semaglutide	Cryo-EM	Full	Active	Gαs, Gβ1, Gy2	Nb35, AIB, WF1	07/21	Zhang et al. 2021
7DUQ	Compound 2	Cryo-EM	Full	Active	Gαs, Gβ1, Gy2	Nb35, Cholesterol, HNO	07/21	Cong et al. 2021
7DUR	Compound 2 (positive ago- allosteric modulator)	Cryo-EM	Full	Active	Gαs, Gβ1, Gy2	Nb35, Cholesterol, HNO(N-tert-butyl-6,7-bis(chloranyl)quinoxalin-2-amine)	08/21	Cong et al. 2021

7EVM	Compound 2	Cryo-EM	Full	Active	Gas, Gβ1, Gy2	Nb35, Cholesterol, HNO(N-tert-butyl-6,7-bis(chloranyl)quinoxalin-2-amine)	08/21	Cong et al. 2021
7KI1	Taspoglutide	Cryo-EM	Full	Active	Gas, Gβ1, Gy2	Nb35, AIB	08/21	Zhang et al. 2021
7E14	LY3502970 or OWL833 (orally active non-peptide agonist)	Cryo-EM	Full	Active	Gas, Gβ1, Gy2	Nb35, Cholesterol, HNO(N-tert-butyl-6,7-bis(chloranyl)quinoxalin-2-amine)	08/21	Cong et al. 2021
7RTB	Peptide 19 (GLP-1R GIPR dual agonist)	Cryo-EM	Full	Active	Gas, Gβ1, Gy2	Nb35, AIB	10/21	Johnson et al, 2021
7LLY	Oxyntomodulin	Cryo-EM	Full	Active	Gas, Gβ1, Gy2	Nb35, AIB	01/22	Wootten et al., 2022
7LLL	Exendin-4	Cryo-EM	Full	Active	Gas, Gβ1, Gy2	Nb35, AIB	01/22	Wootten et al., 2022
7S3I	Ex4-D-Ala (conformer 2)	Cryo-EM	Full	Active	Gas, Gβ1, Gy2	Nb35, AIB	01/22	Belousoff et al., 2022
7S1M	Ex4-D-Ala (conformer 1)	Cryo-EM	Full	Active	Gas, Gβ1, Gy2	Nb35, AIB	01/22	Cary et al., 2022
7VBI	Non-acylated tirzepatide	Cryo-EM	Full	Active	Gas, Gβ1, Gy2	Nb35, AIB	03/22	Zhao et al., 2022
7FIM	Tirzepatide	Cryo-EM	Full	Active	Gas, Gβ1, Gy2	Nb35, AIB	03/22	Zhao et al., 2022
7RGP	Tirzepatide	Cryo-EM	Full	Active	Gas, Gβ1, Gy2	Nb35	04/22	Sun et al., 2022
7RG9	Peptide free	Cryo-EM	Full	Inactive	Gas, Gβ1, Gy2	Nb35	04/22	Sun et al., 2022
7VBH	Triagonist peptide 20	Cryo-EM	Full	Active	Gas, Gβ1, Gy2	Nb35, AIB	04/22	Zhao et al., 2022
7X8S	WB4-24	Cryo-EM	Full	Active	Gas, Gβ1, Gy2	Nb35	06/22	Cong et al., 2022
7X8R	Boc5	Cryo-EM	Full	Active	Gas, Gβ1, Gy2	Nb35	06/22	Cong et al., 2022
7S15	Danuglipron (Pfizer small molecule agonist)	Cryo-EM	Full	Active	Gas, Gβ1, Gy2	Nb35	06/22	Liu et al., 2022
8JIR	GLP-1R/GCGR dual agonist SAR425899	Cryo-EM	Full	Active	Gas, Gβ1, Gy2	Nb35	09/23	Yang et al., 2023

8JIP	GLP-1R/GCGR dual agonist MEDI0382	Cryo-EM	Full	Active	G α s, G β 1, G γ 2	Nb35	09/23	Yang et al., 2023
8JIS	GLP-1R/GCGR dual agonist peptide-15	Cryo-EM	Full	Active	G α s, G β 1, G γ 2	Nb35	11/2023	Yang et al., 2023
8WG7	Peptide free Gs coupled	Cryo-EM	Full	Intermediate	G α s, G β 1, G γ 2	Nb35	03/24	Cong et al., 2024

Despite recent advances in cryo-EM technology, its resolution remains low compared to X-ray structures. This limitation hinders GLP-1R structure modelling in certain receptor regions, such as the cytoplasmic half of TM6 and the stalk between TM1 and ECD, where the density is less defined (295-297). The flexibility and mobility of the ECD section often makes it challenging to resolve, limiting our understanding of interactions in these areas (295-297). Computational approaches like Alpha Fold, a novel machine learning method, can potentially address these limitations (298). By predicting the structures of poorly resolved regions from amino acid sequences, Alpha Fold can create more accurate models with atomic precision. Some structural biology studies have used Alpha Fold to aid in interpreting cryo-EM maps (299-301). However, it is important to note that the accuracy of these missing regions is low according to the per-residue confidence scores. Therefore, any inferences drawn based on the predicted structures should be cautiously interpreted.

Activation and binding

Determining the full active state structure of the GLP-1R has substantially impacted our understanding of the molecular basis of GLP-1R signalling and pharmacology. The elucidation of the active-state GLP-1:GLP-1R:G α s complex structure with near atomic resolution has revealed key residues involved in orthosteric agonist binding and several critical interactions of the receptor with heterotrimeric G proteins. This provides crucial insights into

the molecular mechanisms and conformational changes that occur upon ligand binding, receptor activation, and G protein recruitment.

GLP-1R, like other class B1 secretin-like receptors, typically binds peptide ligands following the 'two-domain' model. The N-terminal ECD, which contains six conserved cysteine residues, serves as the main energy source for this binding mechanism (297, 302). First, the receptor ECD forms multiple interactions with the C-terminal helical region of the endogenous peptide agonist GLP-1. Next the N-terminal end of the GLP-1 peptide forms an extended helical turn, which interacts with and activates the core TMD bundle, triggering receptor activation and G protein engagement (270, 297). Moreover, the GLP-1 peptide is held in place by an extensive network of residue-peptide interactions, as confirmed by mutagenesis studies (302). The GLP-1 binding pocket residues are located deep within the TMD bundle, involving an extensive network of interactions including TMDs 1,2,5,7, ECL1 and 2 (297, 303, 304) (Figure 1.7). The two main GLP-1 residues interacting with the TMD core are His7 and Ala8, which form extensive hydrogen and hydrophobic bonds with TMDs 3,5 and 7, respectively (297, 303, 304). Critical structural waters also anchor the peptide in place by forming a network with His7 on the peptide and TM5 on the receptor (287, 303).

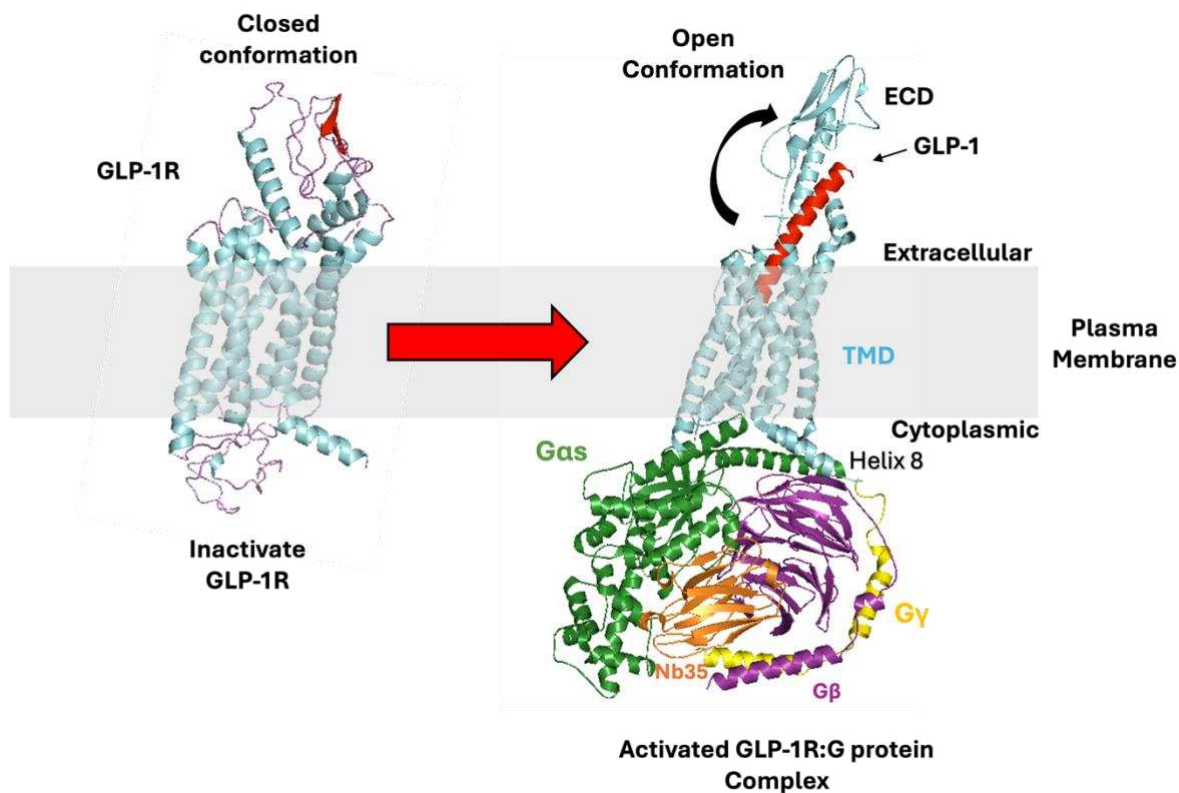


Figure 1.7 The Activation and GLP-1 Binding Mechanism of the GLP-1R. Active vs inactive GLP-1R structures including on the left the inactive GLP-1R:PF06372222 NAM structure (PDB 6LN2) and on the right the active GLP-1R: GLP-1 complex (PDB 6X18).

Recent studies have provided further significant insights into the activation mechanism of GLP-1R, challenging the traditional 'two-domain' hypothesis. Cong *et al.* have presented cryo-EM structures of GLP-1R in conjunction with G proteins, but in the absence of any ligands (305). These ligand-free complexes reveal a $G\alpha_s$ mediated GLP-1R activation mechanism, whereby the $G\alpha_s$ protein mobilises the intracellular TMD binding cavity, altering the extracellular domain to form a transitional intermediate state, and thus facilitating the entry and binding of the peptide at the N-terminus and a fully active state (305). The 'hidden agonist' hypothesis for GLP-1R activation, proposed by Dong *et al.*, suggests another unique

mechanism of GLP-1R activation (278). As per this hypothesis, the GLP-1R has an intrinsic, 'hidden' agonist within its structure (the WDN sequence motif) in the extracellular N-terminal domain (277, 278). This agonist is released upon ligand-induced conformational changes, transitioning the receptor from an auto-inhibited state to an auto-activated state (277). Further mutagenesis studies on the GLP-1R ECD and three ECLs confirm the prominent role of the ECD in GLP-1R trans-activation and support this intrinsic agonist model (305). These novel insights could have significant implications for the development of new GLP-1R targeting therapeutics.

One of the major conformational events induced by peptide binding and TMD interaction is the disruption of polar interactions in the HETx motif (TM2-6-7-helix8) triggering a conformational rearrangement and the opening motion of the cytoplasmic TM6, which in turn activates the receptor and initiates $G\alpha_s$ recruitment (287, 303). Recruitment of G proteins is primarily facilitated by the ICLs in the GLP-1R. Yet, different TMD interactions and orientations are associated with differential coupling to G proteins and inherent signal bias. For instance, the cytoplasmic region of GLP-1R, including the N-terminal side of ICL3, TM7, and Helix 8, interacts with the $\alpha 5$ helix of the $G\alpha_s$ protein. In contrast, $G\alpha_{i/o}$ coupling is mediated by the C-terminal side of ICL3 (270, 297). Biased agonists likely target and modify these regions to favour one G-protein signalling pathway over another.

Different agonists cause unique structural rearrangements and orientations when binding to the receptor, mediating differential receptor intracellular signalling and trafficking. Alanine scanning mutagenesis and modelling studies have uncovered differences in the binding mode of the pharmacological agonist exendin-4 compared to GLP-1 (302). Specifically, exendin-4 has a greater affinity for the receptor ECD due to an extra C-terminal motif, while GLP-1

exhibits more interactions with the receptor core domains (302). Moreover, contrary to the presumption that liraglutide, an acylated long-acting GLP-1 analogue, binds in a similar manner to native GLP-1, *in silico* studies have revealed that liraglutide's side chains point inside the receptor without disturbing the binding site, unlike GLP-1, which occupies the length of the site (306). Similarly, semaglutide also shows slight differences in binding compared to GLP-1. The substitution of Ala8 by aminoisobutyric acid (Aib) in semaglutide results in distinct hydrophobic interactions and water-mediated hydrogen bonds with TM7 and TM3, respectively, compared to GLP-1 (287, 303, 307).

Non-peptide GLP-1R agonists and allosteric modulators have also demonstrated differential GLP-1R binding mechanisms. Compound 2, a GLP-1R positive allosteric mediator (PAM), mediates peptide binding and positive allosteric action by covalently binding with receptor residue C347 within a unique binding pocket formed by ECL1 and 2 near the TM5 and 6 helices (308). This triggers the outward movement of TM6, hydrophobic interactions between key aromatic residues, and the downward shift of the N-terminal α helix of the ECD (287, 308, 309). On the other hand, orfoglipron (LY3502970), an oral non-peptide GLP-1R agonist in development by Eli Lilly, binds in another distinct pocket in the upper helical bundle of the receptor, interacting with residues in the ECD, ECL2, and TM helices 1, 3, and 7, compared to the GLP-1 which binds residues in all TM segments (287, 310).

1.4.4 GLP-1R localisation and role in different tissues

The GLP-1R, crucial to various physiological processes, is found in a variety of tissues throughout the human body. These include the pancreas, nervous system (brain and peripheral nervous system), cardiovascular system, lungs, GI tract, immune system, adipose tissue, eyes,

and the thyroid (Figure 1.8) (311). Localisation of GLP-1R is crucial to understand if the effects of GLP-1 action are direct or indirect. However, many tissue and cellular targets of GLP-1 action such as the liver do not exhibit robust or detectable GLP-1R expression. There are technical challenges in generating reliable and specific anti-GLP-1R antibodies for use in immunoblotting and immunohistochemistry (IHC), which, together with the fact that detection of GLP-1R is method dependent and subject to misinterpretation (311), results in some uncertainty in the precise localisation of the GLP-1R. Therefore, the exact cellular and tissue-specific localisation of GLP-1R *in vivo* is still controversial and poorly understood.

Currently, GLP-1R levels in tissues can be detected through several methods: qPCR for receptor expression levels, immunostaining with validated specific antibodies, such as Mab352, for visualisation and quantification of protein expression (311, 312), receptor mouse models such as GLP-1R CRE:mTmG reporter mice and GLP1R^{SNAP/SNAP} mice for comparing promoter activity with protein levels in specific cells (313, 314), and fluorescent agonists and antagonists (Ex-4-Cy3 and -Cy5, and Luxendins) (228, 312, 313) for live tissue imaging and visualisation of GLP-1R capable of ligand binding and activation.

The expression patterns of the GLP-1R in rodent and human pancreatic islets have been studied extensively using multiple methods including transcriptional analysis of sorted islet populations, enriching endocrine cell populations with genetic reporter lines followed by bulk RNA sequencing to analyse enriched cell populations from reporter mice, qPCR and scRNA-seq (311). Early studies using the above methodologies revealed that within pancreatic islets β -cells robustly express GLP-1R (80-95%), while δ -cells exhibit moderate expression and α -cells low to undetectable levels (0-10%) (315-318). Yet, studies assessing heterogeneity in GLP-1R expression within islet endocrine cell populations have revealed variability in GLP-

1R expression between individual β - and δ -cells and a subpopulation of unique α -cells (1%) with significantly more *InsI* expression, that are also GLP-1R-positive (314). This small subpopulation of α -cells expressing GLP-1R was further validated (228). All in all, it is crucial to consider the limitations associated with these detection methods (312). Therefore, it is recommended that future research combine different approaches and validate findings in tissues lacking GLP-1R, to detect the receptor more accurately. Nevertheless, generation of validated membrane-specific GLP-1R antibodies and effective fluorescent agonists and antagonists has allowed us to validate receptor localisation more reliably in non-pancreatic cells and tissues.

The key role that GLP-1 derived from PPG or GCG neurons plays in hypophagic and reduction of food intake responses to GLP-1R agonists, suggests the possible presence of the receptor in the nervous system (311, 319), where the GLP-1R is found throughout the CNS, from the olfactory bulb to the spinal cord, as well as in the sympathetic nervous system where it plays a role in stress induced responses (228, 238, 314, 320-324). In the brain, the GLP-1R is detected in glutamatergic neurons and neurons of the NTS, where activation of the receptor is believed to modulate liraglutide anorectic and hypophagic effects by signalling to GABAergic neurons and triggering the release of inhibitory neurotransmitters (325, 326). The GLP-1R is also localised in circumventricular organs [area postrema (AP), median eminence and ARC], structures that are localised around the brain ventricles lacking a blood brain barrier and sense blood derived molecules such as polypeptide hypothalamic hormones (238, 324, 327-329). Some of these areas are innervated by PPG neurons and studies suggest that GLP-1R activation in these areas is induced by high blood GLP-1 levels as a result of severe intestinal malaise or post bariatric surgery (311). Despite inconsistent patterns of GLP-1R expression in rodents,

studies have reported high levels of GLP-1R expression in the mouse hippocampus and receptor responses to GLP-1R agonist administration (324, 329). Electrophysiological studies and behavioural studies reveal that GLP-1-induced GLP-1R activation mediates depolarisation of neurons in ventral hippocampal regions reducing food intake, body weight and food motivated behaviours (324, 330). Activation of GLP-1R in hippocampal neurons promotes proliferation and survival improving cognitive function and reversing memory impairment (331, 332) (317,318). Lastly, GLP-1R is also expressed in the parasympathetic nervous system, specifically in vagal afferent neurons, where they play a role in the gut-brain axis and slowing down gastric emptying. Vagal afferent neurons are a set of mechanoreceptors constituting a small fraction of vagal afferent inputs to PPG neurons, with cell bodies in the nodose ganglion and axon terminals in the NTS (314, 333, 334). The exact localization of GLP-1Rs is unclear, but GLP-1R knockdown experiments using shRNA in the nodose ganglion of male rats have shown accelerated gastric emptying, and impaired response to exendin-4 on food intake (328, 335). This implies that vagal afferent neuron GLP-1R expression is localised to the peripheral nodose ganglion rather than nerve terminals inside the NTS.

Consistent with the detection of GLP-1R transcripts and protein in the human heart and blood vessels, GLP-1R exerts multiple beneficial effects in the cardiovascular system, including regulation of heart rate (336), vasodilation, and reduction of blood pressure during hypertension (337). GLP-1R expression has been detected in various parts of the cardiovascular system including sinoatrial myocytes, sinoatrial node, right atrium, and ventricle, as well as in atrial and ventricular cardiomyocytes (338, 339). As with other tissues, there is tissue specific heterogeneity in receptor expression, whereby GLP-1R expression and

mRNA levels are more prevalent in atria compared to ventricles, specifically in the right atrium. GLP-1R is also detected in blood cells and arterial circulation including aorta, arteries, arterioles and arterial pericytes (311). Yet here also receptor expression is highly heterogeneous, with high expression in mouse lung endothelial cells and restricted expression in subsets of renal vascular smooth muscle cells (311). Although studies have shown that GLP-1R activation in the arterial system prevents the development and progression of coronary atherosclerosis, regulates arterial blood pressure, induces vasodilation and increased blood flow in the peripheral circulation (337, 340, 341), mechanisms by which GLP-1R modulates renal and lung function remain less well understood (342).

Other tissues where the receptor is detected include immune system cells (T and invariant natural killer T (iNKT) cells and macrophages) (343-345), gastrointestinal tract (parietal cells, Brunner's gland, enteric nervous system, and intraepithelial lymphocytes) (346-348), adipose tissue (349, 350), thyroid C cells (351), and retinal ganglion cells (352). Although, the role of GLP-1R in the immune system function is increasingly studied, the role in other tissues is less well defined. GLP-1R agonist administration exerts anti-inflammatory actions via reducing the production of pro-inflammatory cytokines in autoimmune diseases like asthma (353).

In conclusion, the diverse distribution of the GLP-1R across tissues reflects its multifaceted role in physiological processes. Therefore, more research is needed to accurately map its localisation and function in specific cellular populations and tissues.

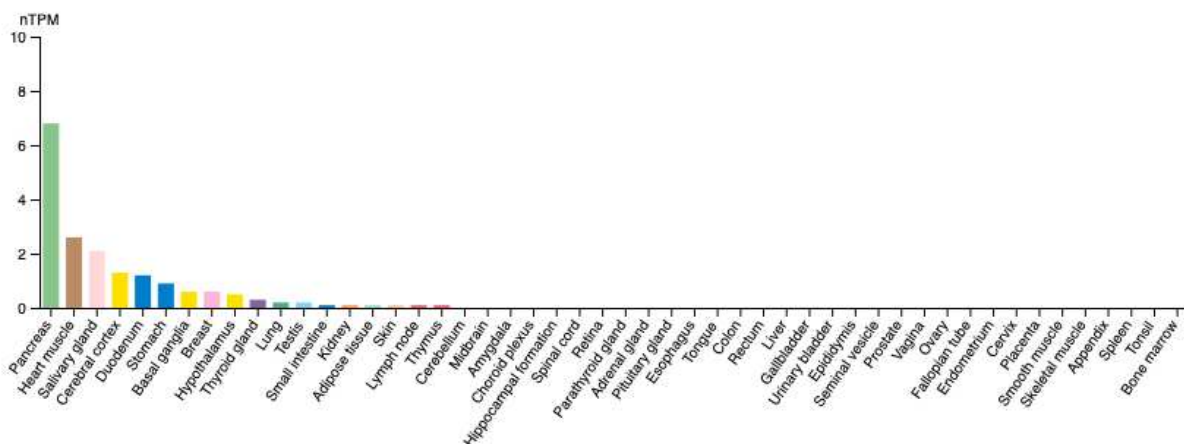


Figure 1.8 Overview of GLP-1R mRNA expression in different tissues. Data taken from the consensus dataset of the human tissue atlas server. The consensus dataset consists of normalized expression (nTPM) levels for 55 tissue types, created by combining the HPA and GTEx transcriptomics datasets using the internal normalization timeline. Color-coding is based on tissue groups, each consisting of tissues with functional features in common.

1.4.5 GLP-1R trafficking and signalling: effects on insulin secretion and glucose homeostasis

Trafficking

GPCR trafficking mechanisms are crucial regulators of signalling capacity and can be manipulated to enhance pharmacological agonist effects (Figure 1.9). Binding of ligands to their corresponding GPCRs initiates signalling and simultaneously promotes the activation of feedback mechanisms to regulate the receptor response. These include trafficking mechanisms such as internalisation, recycling, and degradation to regulate re-sensitisation and desensitisation of the receptor (354, 355). These mechanisms are crucial in cases of chronic or repeated stimulation, protecting the receptor from overstimulation, despite posing limitations for therapeutic development (354, 355). GLP-1R trafficking pathways resemble canonical GPCR ones, whereby the ligand-activated receptor internalises from the plasma membrane

into intracellular compartments where it passes through a complex network of endosomal trafficking pathways encompassing key internal subcellular compartments such as early, recycling, and late endosomes (multivesicular bodies), lysosomes, as well as retrograde transport to the TGN and endoplasmic reticulum (ER) (356, 357).

The exact molecular mechanisms underlying GLP-1R internalisation are still unclear, as there is evidence in support of two main GPCR endocytosis pathways, clathrin-coated pits and caveolae, resulting in inconsistent findings (358-360). Although the exact pathway is still under debate, there is strong evidence to suggest that GLP-1R internalisation via either pathway is dynamin dependent as inhibition using a dominant negative form (dynamin-1 K44E), impairs agonist-mediated GLP-1R internalisation completely in FlpIn-CHO cells (307). The internalisation pathway of GLP-1R may be context-dependent; the receptor may be utilising a different mechanism based on the specific ligand bound or cell type. For instance, Thompson and Kanamarlapudi showed that in HEK293 cells internalisation of the GLP-1R was inhibited by defective caveolin pathway rather than clathrin-dependent pathways (359). Whereas, in a murine pancreatic β -cell line (MIN6B1) lacking detectable caveolin-1 levels, gene silencing of factors involved in the formation of clathrin-coated pits such as AP2 and clathrin, increased incretin dependent insulin secretion as a result of decreased receptor internalisation (361). This suggests that although the receptor favours one pathway, it can use endocytosis mechanisms interchangeably, potentially upregulating one pathway to compensate for the loss of the preferred one, depending on cell specific machinery. Thus, this explains the challenge in validating the main endocytic route employed by the receptor. Moreover, other factors like plasma membrane composition, specifically membrane

cholesterol levels as well as alterations in receptor structure may also influence these mechanisms.

Traditionally, it was believed that GLP-1R internalisation and desensitisation were dependent on β -arrestin recruitment. In contrast, more recent studies have contradicted this showing evidence that β -arrestin 2 knockout in cells and animal models does not inhibit internalisation (357, 362). In the same vein, a recent study by McNeill. *et al.* showed that GLP-1R internalisation is independent of β -arrestin1/2 recruitment and is instead dependent on expression of GRKs, overexpression of which rescues both β -arrestin 1 recruitment and internalisation profiles in HEK293 cells (363). Others have also argued that GLP-1R internalisation is mediated by activation of the $G\alpha_{q/11}$ pathway and its downstream signalling effectors, PKC and ERK (359). Overall, additional work is required to understand how GLP-1R internalisation is controlled in different conditions.

Following internalisation, GLP-1R is initially directed to early endosomes (Rab5+), where it can subsequently be directed through various trafficking routes, including recycling and degradation pathways (356, 364-366). Receptors can be recycled to the plasma membrane initiating re-sensitisation by passing via two pathways: the short cycle (Rab4+ recycling endosomes) and the long cycle (Rab11+ perinuclear recycling endosomes) (227, 364, 365, 367, 368). Conversely, receptors can also be directed into multivesicular bodies / late endosomes (Rab7+) and eventually targeted to lysosomes for degradation and signal termination (227, 356, 364). Endocytic recycling of GPCRs to the plasma membrane is believed to be regulated by retromer complexes containing sorting nexin 27 (SNX27) interacting with cargo GPCRs via their PDZ domains (369). Indeed, an RNAi screen in MIN6B1 β -cells revealed that the sorting nexins SNX1 and SNX27 control the balance

between GLP-1R plasma membrane recycling and lysosomal degradation, determining overall β -cell responses to GLP-1R agonists (361). Ligand-activated GPCRs such as the β_2 -adrenergic receptor (370), μ -opioid receptor (371), and V2 vasopressin receptor are efficiently targeted for lysosomal degradation by ubiquitination, in a mechanism involving β -arrestin 2 dependent NEDD4 recruitment and ubiquitin-specific binding to the endosomal sorting complex required for transport (ESCRT) machinery (372). In parallel to this, Bitsi. *et al.* (2023) showed constitutive GLP-1R NEDD4 recruitment and ubiquitination, with exendin-4 stimulation triggering partial de-ubiquitination of the receptor (362).

The route and timing/kinetics of endocytosis and endosomal trafficking of receptors are dependent on several factors including the ligand, alterations in receptor structure and plasma membrane composition. For instance, studies investigating the real-time internalisation of GLP-1R for the first time revealed that GLP-1R internalises rapidly and is a fast-recycling receptor, exhibiting similar rapid internalisation rates but faster recycling rates when bound to GLP-1 relative to exendin-4 and liraglutide (365). In another analysis of agonist specific GLP-1R internalisation and trafficking profiles, Jones *et al.* (2018) used the SNAP-tag system in CHO-K1, INS-1 832/3 and MIN6B1 β -cells to show that exendin-4 and its β -arrestin 2 biased analogue exendin-asp3 (exendin-D3) exhibit fast-internalisation relative to the cAMP biased analogue exendin-phe1 (exendin-F1), resulting in longer presence at the plasma membrane, reduced desensitisation and enhanced insulin secretion with the latest agonist (357). In parallel to this, exendin-F1 also exhibited faster recycling and reduced degradation compared to its analogues exendin-4 and exendin-D3 (343). Likewise, Novikoff *et al.* (2021) also assessed internalisation and trafficking dynamics of GLP-1R agonists like semaglutide as well as dual agonists like tirzepatide using bioluminescence resonance energy transfer (BRET) based

assays (373). They revealed that the dual GLP-1R/GIPR agonist tirzepatide exhibited diminished internalisation at the GLP-1R while maintaining localisation to Rab11 endosomes and recycling profiles relative to the GLP-1R monoagonist semaglutide (373). In light of these findings, it is clear that agonist-specific endocytosis and recycling influences surface GLP-1R levels, pharmacological insulin secretion and glycaemic benefits.

Signalling

Akin to other GPCRs, upon activation by its ligand, a conformational change occurs in the GLP-1R stabilising it and enabling coupling to occur with heterotrimeric guanine nucleotide-binding proteins (G proteins), which consist of three distinct subunits (α subunit and a β/γ dimer) (374, 375). The $G\alpha$ subunit is further subdivided into four families based on their distinct signalling profiles: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$ (376). The activated GLP-1R exhibits complex signalling as it is pleiotropically coupled, signalling via several G protein dependent and independent mechanisms (Figure 1.9).

In pancreatic β -cells the GLP-1R mediates its physiological effects via various downstream signalling pathways including cAMP, intracellular calcium signalling, and the phosphorylation of MAPK pathways (Figure 1.9) (293, 375, 377). In the presence of high glucose, activated GLP-1R preferentially couples with the $G\alpha_s$ protein, activating AC-mediated cAMP accumulation, and subsequently cAMP-mediated signalling pathways including its downstream targets PKA and Epac2 (293, 377). The activation of the PKA pathway and the translocation of Epac2 to the cytoplasmic membrane augments membrane depolarisation and intracellular calcium influx by phosphorylation of SUR1 KATP channel subunit, which, combined with insulin granule priming enhances insulin granule mobilisation and exocytosis (377). Additionally, direct cAMP activity and PKA dependent CREB

activation alter gene expression, enhancing transcription of the pro-insulin gene as well as genes such as *Irs-2* improving insulin secretion, and β -cell proliferation and survival, respectively (378, 379). Ligand-activated GLP-1R can also signal via other G proteins, such as $G_{q/11}$, G_{α_i} , and G_{α_o} , leading to activation of phospholipase C (PLC), inducing intracellular calcium mobilisation, activation of ERK1/2 and Akt pathways, and phosphorylation of PIK3, amongst other readouts (377, 379). As for G protein independent pathways, ligand-binding associated conformational changes enabling phosphorylation of TM7 by GRKs is crucial for the recruitment of β -arrestins by the receptor promoting G protein uncoupling and desensitisation as well as ERK1/2 activation, β -cell proliferation, and insulin secretion by β -arrestin-dependent signalling (293, 362, 377, 379).

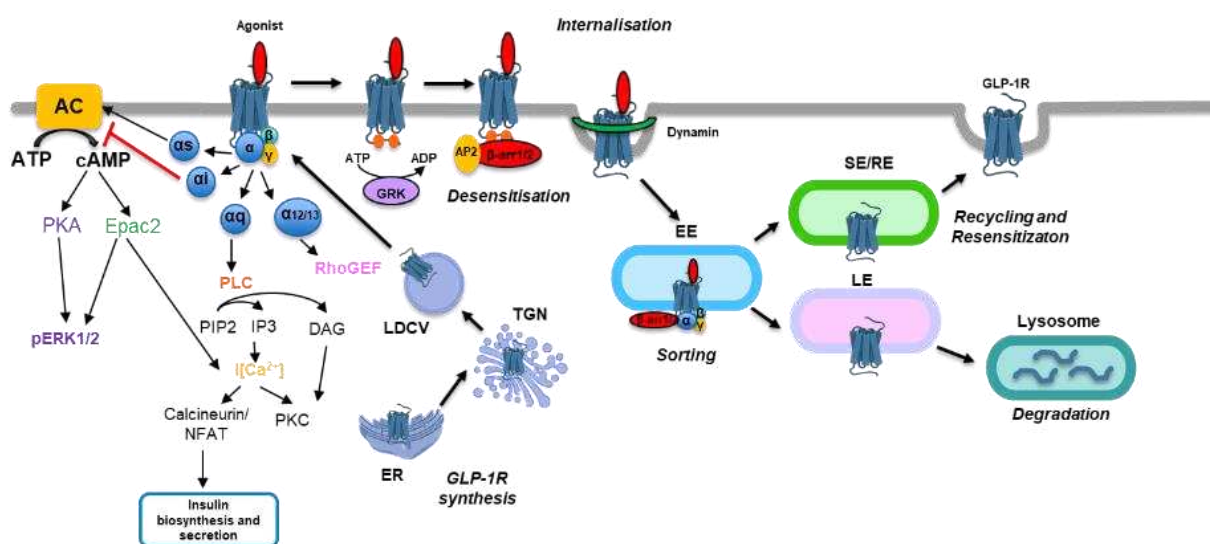


Figure 1.9 Main pathways of trafficking and signalling of the GLP-1.

1.4.6 GLP-1R biased and spatiotemporal regulation of signalling

Biased signalling

GLP-1R pleiotropic signalling is further complicated by the fact that the receptor can be activated by various peptide and non-peptide ligands, permitting biased signalling to occur. Biased signalling, a pharmacological paradigm originally coined by Jarpe *et al.*, refers to a concept whereby different ligands are able to selectively activate distinct signalling pathways and downstream effectors upon engagement with the same receptor (380). In recent years, there has been increasing amounts of literature on biased agonism in drug discovery for T2D and obesity, as it has the potential to improve existing therapies, enhancing benefit-to-adverse effects ratios, as well as explaining differences in clinical outcome profiles of different GLP-1R agonists (293, 381). The signalling profile across three pathways (cAMP, intracellular Ca^{2+} elevation, and ERK1/2 phosphorylation) of GLP-1, exendin-4 and oxyntomodulin have been well characterised in Chinese hamster ovary (CHO) cells expressing GLP-1R (309, 382). These studies show that these three agonists preferentially activate the $\text{G}\alpha_s$ -mediated-cAMP pathway over ERK1/2 phosphorylation *in vitro*; yet both exendin-4 and oxyntomodulin are biased towards the ERK1/2 signalling pathway over cAMP production relative to the native GLP-1 (309, 382). In parallel to this, Wootten *et al.* found that exendin-4 and oxyntomodulin are also more effective at recruiting β -arrestin 2 than the native GLP-1 peptide (304). Furthermore, Fletcher *et al.* performed additional studies assessing the signalling profile of these agonists as well as liraglutide in live CHO cells, revealing that liraglutide favours pERK1/2 activation but is biased away from β -arrestin 2 recruitment compared to exendin-4 (383).

Conventionally, physiologically beneficial effects of GLP-1R activation such as insulin secretion were associated with increased activation of $\text{G}\alpha_s$ -mediated cAMP production,

whereas β -arrestin 2 recruitment was associated with receptor desensitisation and reduced GLP-1R agonist efficacy. As a result, researchers sought to develop GLP-1R agonists biased away from β -arrestin 2 recruitment over cAMP production (381). The first compound designed with this in mind was exendin-P5, an exendin-4 analogue with an 8-residue modification at the start of the sequence, identified in a large (>100 million peptide) high throughput screen (384). *In vitro* characterisation of exendin-P5 revealed a $G\alpha_s$ biased signalling profile, with full agonist activity for cAMP and significantly defective β -arrestin 2 recruitment. Despite a promising signalling profile, this peptide exhibited an interesting *in vivo* profile, with a greater acute blood glucose lowering effect than exendin-4 but lower insulin secretion levels (385, 386). Another study also developed and investigated a biased exendin-4 analogue, exendin-F1, featuring a single amino acid substitution from histidine to phenylalanine in the N-terminus (381, 387). This peptide also demonstrated a distinct biased signalling profile, displaying full agonist activity for cAMP production and markedly reduced β -arrestin 2 recruitment and receptor desensitisation (381, 387, 388). Subsequently, this translated to increased insulin secretion and blood glucose lowering effects after prolonged (4-8 hrs) exposure to the peptide compared to exendin-4 in T2D mouse models (381, 389).

Another peptide modification that could result in biased agonism via altered interactions with the receptor structure is the installation of fatty acid moieties to the middle or C-terminus of peptides, to enhance albumin binding and extend pharmacokinetics, such as the GLP-1R agonists liraglutide and semaglutide (390-392). Indeed, a study comparing exendin-4 to an exendin-4 analogue C-terminally attached to a fatty diacid moiety (exendin-4-C16) using high-content microscopy, proximity-based assays, and BRET complementation assays, revealed that addition of this fatty acid diacid altered GLP-1R trafficking and signalling, reducing

internalisation and clustering in membrane nanodomains, as well as reducing ligand binding affinity and β -arrestin 2 recruitment, respectively.

Specific ligand-receptor interactions can stabilise different structural conformational arrangements, influencing the preference for certain signalling pathways. In fact, structural studies using a combination of homology modelling, mutational analysis and MD simulations revealed key residues involved in both peptide-mediated bias and general signalling bias (304, 393). The four key residues (Arg190, Asn240, Gln394 and His363) form a central polar network in the middle of the helical bundle of the receptor influencing cAMP signalling and ERK1/2 phosphorylation (282). Additionally, structural studies superimposing different activated agonist-GLP-1R complexes identified further network interfaces involved in coupling specific signalling pathways such as the TM5 and 6, associated with cAMP and intracellular calcium, while TM1 / TM7 / ECL3 were associated with $G\alpha_i/G\beta\gamma/\beta$ -arrestin 2 and pERK coupling (393). Taken together, these studies show that a combination of structural, mutational and homology studies can be utilised to identify distinct structural reorganisations in bias-associated networks to predict peptide-specific signalling pathways, and subsequently agonist efficacy.

Spatiotemporal compartmentalisation of signalling

Trafficking mechanisms are pivotal for the temporal (duration) and spatial control (localisation) of GLP-1R signalling, a concept known as spatiotemporal compartmentalisation. Spatiotemporal compartmentalisation is another factor that can influence a receptor signalling profile (293, 394). Despite classical theories suggesting a positive correlation between cell membrane expression and receptor efficacy, more recent studies recognise that increased receptor internalisation might translate to sustained G protein signalling from intracellular

locations, prolonging specific receptor responses. Studies are increasingly focusing on the concept of spatiotemporal compartmentalisation of incretin receptors such as the GLP-1R (364, 373, 394).

There is evidence for GLP-1R signalling from intracellular locations such as endosomes, the nucleus and the TGN. Results of recent studies showed extensive co-localisation of GLP-1R with AC and reduced PKA and ERK1/2 phosphorylation, cAMP, and calcium signalling following inhibition of GLP-1R internalisation via a dominant negative dynamin K44E (365, 395). Similarly, a recent study by Manchanda *et al.* used novel bystander NanoBiT complementation assays to assess plasma membrane *versus* endosomal $G\alpha_s$ coupling by the GLP-1R and the GIPR, showing that although both have endosomal activity, GLP-1R exhibits higher endosomal *versus* plasma membrane activity (364). Indeed, endosomal trafficking of the GLP-1R and endosomal cAMP signalling were found to mediate insulin granule exocytosis in pancreatic β -cells (395). Interestingly, a study by Bitsi *et al.* also found that the ligand activated GLP-1R can reroute away from lysosomal compartments to the TGN, where it exhibits significantly increased cAMP levels in cells lacking β -arrestin 2 (362). Moreover, studies have also shown that GLP-1R segregates within cholesterol-rich lipid rafts or plasma membrane nanodomains, which serve as GLP-1R signalling hotspots, via enhanced receptor caveolin interactions increasing agonist binding affinity (358).

Biased GLP-1R agonists can also elicit distinct spatiotemporal profiles of signalling, and this is often associated with their altered trafficking and endocytic profiles. A study by Fletcher *et al.* showed that pERK1/2 biased agonists liraglutide and oxyntomodulin induced both nuclear and cytosolic pERK1/2 activity, whereas GLP-1 and exendin-4 induced more sustained

ERK1/2 signalling restricted to the nucleus, suggesting possible effects on the regulation of β -cell associated gene transcription (379, 383).

1.5 Current therapeutics for T2D: Insulin secretagogues and incretin based therapies

1.5.1 Insulin secretagogues

Insulin secretagogues are antihyperglycemic agents that enhance insulin secretion by regulating various mechanisms in pancreatic β -cells and are extensively used in the treatment and management of T2D. These compounds include sulfonylureas, glinides, incretin-related drugs, glucokinase activators (GKAs), free fatty acid receptor (FFAR) and amino acid receptor agonists (396). Studies using humanised mouse models with functional human islets indicate that these compounds effectively stimulate human C-peptide secretion, and improve postprandial glucose levels, maintain euglycemia, and reduce β -cell dysfunction (397).

Sulfonylureas, clinically in use since the 1950s, were inaugural oral antidiabetic agents. They continue to play a crucial role in glycaemic control due to their rapid glucose-lowering effects, particularly for individuals with high glycosylated haemoglobin A1c (HbA1c) levels (398). Both sulfonylureas and glinides, used as first-line therapy in T2D, operate by closing β -cell KATP channels and increasing calcium ion influx to increase insulin secretion (399, 400). They bind to sulfonylurea receptors (SURs) on the β -cell surface, particularly SUR1, a regulatory subunit which forms the KATP channel with the inwardly rectifying K^+ channel (Kir6.2) (401, 402). Activating SUR1 blocks Magnesium Adenosine Diphosphate (Mg-ADP),

directly inhibiting Kir6.2/SUR1 KATP channels, preventing efflux of potassium ions from the β -cell cytoplasm. This action depolarises the β -cell membrane, opening voltage sensitive calcium ion channels and triggering calcium ion influx from the exterior, leading to insulin exocytosis (400, 402). Some sulfonylureas also cooperatively function with cAMP to mobilise Epac2 in the active form (403, 404), while glinides mobilise calcium from the ER (405). Despite their similar mode of action, glinides differ in pharmacokinetics and receptor binding, exhibit weaker receptor binding affinity and faster elimination by faecal excretion post liver metabolism, resulting in a more rapid onset and shorter duration of action compared to sulfonylureas (406). Despite their beneficial effects in controlling postprandial glucose excursions, reducing glucagon secretion, and enhancing insulin sensitivity in peripheral tissues, these compounds elicit adverse side effects. These include increased risk of fatal hypoglycaemia, cardiotoxicity, weight gain and drug interactions (407-410).

More recently, novel compounds such as glucokinase activators (GKAs), FFAR, and amino acid receptor agonists with insulin secretion propensity have been developed for T2D therapy (396). GKAs function as glucose sensors in glucose-sensitive cells, including pancreatic β -cells, hepatocytes, enteroendocrine cells and hypothalamic glucose-sensing (GS) neurons (411). They catalyse the release of glucokinase from its inhibitory complex into the cytoplasm and the phosphorylation of glucose to glucose-6-phosphate (G6P) (411, 412). However, clinical trials with activators like MK-0941 (AZD1656) have been linked with several adverse effects such as hypoglycaemia, dyslipidaemia, and fatty liver disease (413). Therefore, further studies are necessary to better understand their mechanisms of action and develop improved GKAs without these side effects.

Discovered in 2003, GPR40, a free fatty acid GPCR primarily found in pancreatic β -cells, increases insulin secretion in response to long chain free fatty acids in a glucose dependent manner (414). Due to its insulintropic actions, it has been investigated for T2D therapeutics. This GPR40 is coupled to $G_{\alpha_{q/11}}$ protein, whose activation promotes the hydrolysis of PIP_2 into diacylglycerol (DAG) and IP_3 by phospholipase C (PLC). This triggers the mobilisation of calcium ions from ER stores via phosphokinase C (PKC) activation (414-416). Yet, TAK875, a GPR40 agonist, failed in phase III clinical trials due to hepatotoxicity (417). As a result, additional studies on its mechanisms of action in extra-pancreatic tissues, such as the liver, are needed to generate successful therapeutics. Another GPCR, GPR142, a novel orphan class A GPCR, has been found to simulate insulin secretion by controlling incretin and insulin secretion in the pancreas (418). GPR142 is an aromatic amino acid receptor, specifically activated by tryptophan, which activates $G_{\alpha_{q/11}}$ signalling to induce glucose-dependent incretin control and insulin secretion, reducing the risk of hypoglycaemia (419, 420).

Incretin related therapeutics have also been extensively studied as insulin secretagogues and T2D therapeutics. The incretin hormones, GIP and GLP-1, potentiate insulin secretion at elevated postprandial blood glucose levels, potentiating insulin secretion in a glucose dependant manner (421). Incretin T2D therapeutics are classified into two groups: incretin enhancers (DPP-4 inhibitors) and incretin mimetics (GLP-1R agonists). DPP-4 inhibitors increase the levels of active state GLP-1 and GIP hormones, while GLP-1R agonists directly activate GLP-1R mediated signalling in β -cells (422).

1.5.2 Incretin-based therapies: GLP-1R agonists (GLP-1RAs)

In the mid-1980s, GLP-1 was isolated and characterized by Joel Habener and colleagues at Harvard as a product of post-translational processing of proglucagon in the intestinal L-cells and brain. Subsequently, the active insulinotropic forms of GLP-1, GLP-1(7-36) amide and GLP-1(7-37), were identified and characterised by Daniel J. Drucker, Joel Habener and Steven E. Kieffer. These peptides have since been extensively studied as therapeutic targets for T2D treatment due to their potent insulinotropic action, stimulating insulin secretion in a glucose dependent way upon meal ingestion (423).

Unlike GIP, the other incretin hormone, GLP-1 maintains its activity in T2D patients, eliminating postprandial glucose rises and reducing appetite, food intake, and body weight (250). Early studies employing intravenous (i.v.) and subcutaneous (s.c.) administration of GLP-1 revealed its short half-life in circulation pertaining to its degradation and elimination by DPP-4 (215, 424), thus, necessitating the generation of modified versions of this peptide with slower elimination kinetics and DPP-4 resistance for clinical use.

GLP-1R agonists (GLP-1RAs), injectable insulin secretagogues that are GLP-1 analogues, are used in the clinic as second line monotherapies or in combination with other therapies in dual, triple, or more complex regimens (425). Currently approved GLP-1RAs include exenatide, liraglutide, albiglutide, dulaglutide, lixisenatide and the recently added semaglutide, with more in development (Figure 1.10) (425, 426). Indeed, recent studies have also recommended using GLP-1RAs as first-line therapies in patients with established cardiovascular disease, high risk of cardiovascular disease, chronic kidney disease or heart failure (427, 428). Equally, in view of the significant reduction in weight and appetite observed in patients administered these

GLP-1RAs, they have been recently approved and highly recommended for chronic weight management in adults with obesity both in the UK and in the US (429).

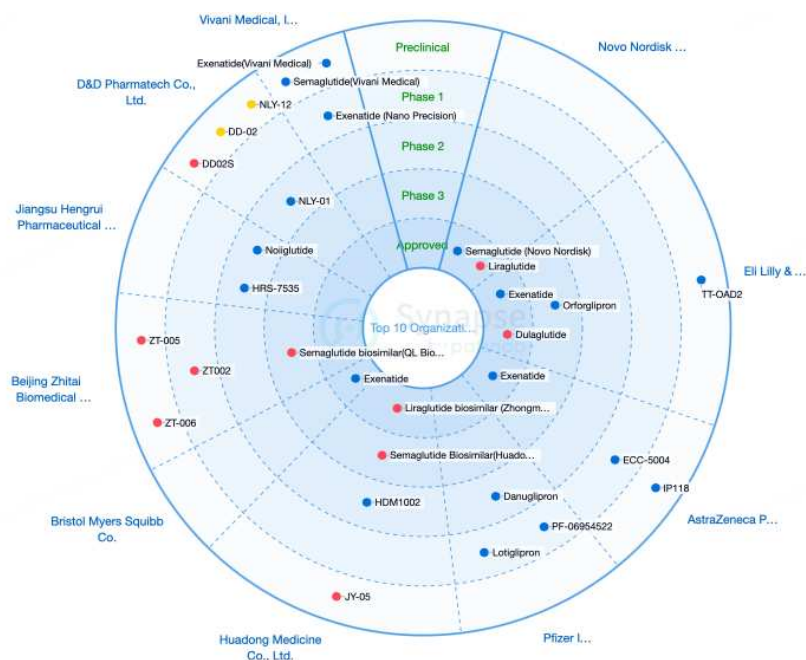


Figure 1.10 List of all available GLP-1R agonists in the clinic and in development. Details include trial stage, name and biopharmaceutical company obtained from Synapse by patsnap server.

GLP-1RAs are classified into two categories: short-acting and long-acting, depending on their time-action profile and half-life (430). For instance, exenatide and lixisenatide, which show rapid rises in plasma drug concentrations after subcutaneous administration, are considered short-acting agonists. Typically, they are prescribed once or twice daily prior to meals to reduce postprandial glucose levels (425, 428). They also significantly decelerate gastric emptying reducing plasma triglyceride and fatty acid levels (431). On the other hand, long-acting GLP-1RAs such as liraglutide, albiglutide, dulaglutide and semaglutide, maintain their plasma concentrations for an extended period, remaining effective for at least 24 hours after administration (431, 432). Due to high plasma drug concentrations between doses, GLP-1R is

continuously activated, and thus long-acting agonist effects are mediated mainly through the receptor actions on GSIS and glucagon secretion inhibition, resulting in enhanced glycaemia throughout the whole day, including during fasting intervals (431-433). Unlike the short-acting agonists, these drugs affect both fasting and postprandial glucose levels, controlling glycemia more effectively and lowering HbA1c levels (432).

The first GLP-1RA approved for T2D treatment was exenatide (Byetta), a synthetic version of exendin-4. The exendin-4 peptide was discovered serendipitously in 1992 by Dr. John Eng, an endocrinologist at the Veterans Affairs Medical Centre in Bronx, who identified it in the saliva of the venomous lizard the Gila monster (*Heloderma suspectum*), along with its fragment that acts as an antagonist (434). This peptide has 53% structural similarity to the GLP-1 hormone and similar binding affinity to the GLP-1R. Importantly, it also exhibits resistance to degradation by DPP-4, allowing it to be eliminated more slowly with a half-life of 2-3 hours (434, 435). This led to its approval for twice-daily subcutaneous injection in 2005, typically administered prior to breakfast and dinner.

The next GLP-1R peptide agonist to be approved for daily subcutaneous injections in 2009 was liraglutide. It was developed as a GLP-1 backbone derivative with a 97% similarity to the native GLP-1 hormone (436). Its action was prolonged by the attachment of a free fatty acid chain via linker molecules, promoting albumin binding in extracellular fluid and plasma. Accordingly, only 1-2% of non-albumin bound liraglutide exists in circulation, with the remaining peptides making up an albumin reservoir that dissociates to reach GLP-1R expressing tissues and cells (436). Due to its long action, liraglutide has a half-life of 13 hours and is approved for once-daily use in the clinic (427, 437).

To extend the half-life and duration of action of exenatide (the un-retarded twice-daily compound), the peptide was encapsulated in dissolvable polymer microspheres, which remain in place under the skin and slowly dissolve following subcutaneous injection, releasing active exenatide into the blood (437, 438). This ensures exenatide plasma concentrations slowly and continuously rise to reach a steady state within about 10 weeks, meaning it takes weeks for all the exenatide to be absorbed and eliminated even after discontinuation of the treatment (439). This polymer encased exenatide was the first compound designed and approved for once weekly injections. In randomised clinical trials, exenatide once weekly was associated with significant glycaemic enhancements and moderate weight loss when administered as monotherapy or in combination with other oral antidiabetic agents in diabetic patients (438, 440).

Dulaglutide (Eli Lilly) and albiglutide (Glaxo SmithKline), two other GLP-1R peptide agonists approved for once-weekly injections, had their life extended by attaching large proteins such as the immunoglobulin Fc fragment (peripherally) or albumin (incorporated into the linear sequence), respectively (441, 442). The attachment of these large carrier proteins significantly delays the degradation and elimination of these compounds from circulation, resulting in one-week half-lives (441-443). Not only slow elimination, but also rapid absorption leading to earlier onset of clinically noticeable beneficial effects, means it surpasses the exenatide once-weekly compound in terms of rapid glycaemic enhancements (444).

Semaglutide (Wegovy), the most recent once-weekly GLP-1R peptide monoagonist to be approved, developed by Novo Nordisk, has had unprecedented success in both T2D and obesity management in the clinic (445). Results from the recent SUSTAIN-7 clinical trial showed that semaglutide once-weekly outperformed dulaglutide and liraglutide at both low

and high doses in improving glycaemic control and weight loss, as well as having a high adherence rate (446). This compound is also a long-acting agent, resembling liraglutide in structure, except for a modification substituting a crucial DPP-4 recognition site (alanine residue at the 8th position) for 2-aminoisobutyric acid (Aib), protecting it from enzymatic degradation by DPP-4. Moreover, this modification also tightens the binding of the fatty acid side chain, augmenting the elimination delay and resulting in half-life of a week (445, 447).

To improve patient adherence and treatment tolerability, an oral preparation of semaglutide (Rybelsus) has been developed by Novo Nordisk and has been FDA approved for T2D and obesity management. This oral version contains an additional absorption enhancer, sodium N-(8-(2-hydroxybenzoyl) Amino) Caprylat (SNAC), which raises the local pH, preventing semaglutide degradation and facilitating absorption via the gastric mucosa (448). This quickly popular therapeutic agent has demonstrated superior and clinically relevant improvements in HbA1c levels and significant weight loss in T2D patients similar to those elicited by the injectable semaglutide in clinical trials (448, 449). However, a higher ingested dose was required to achieve similar plasma concentrations as the subcutaneously administered semaglutide (14 mg daily compared to 1 mg per week) accounting for lower bioavailability due to gastrointestinal absorption (450). This, as well as the requirement to ingest it in the fasted state with no consumption of food or liquid for at least 30 minutes after, might lower its tolerability profile. Nevertheless, it still is the first GLP-1R agonist approved for oral administration and that is an impressive achievement.

Notwithstanding the various beneficial effects of GLP-1R agonists in managing several diseases such as diabetes, obesity, cardiovascular disease, and more recently, neurodegenerative diseases, their use is still limited by adverse effects that can affect patient

adherence. Since GLP-1RAs mimic the action of the GLP-1 hormone produced in intestinal L-cells, most reported side effects relate to the GI tract, including nausea, vomiting, and diarrhoea that could lead to acute kidney injury due to fluid loss (451). Other non-GI associated side effects have also been reported, including dizziness, mild tachycardia, infections, headaches, and dyspepsia, but these are much rarer (452, 453). GI adverse reactions (GIARs) are most prominent at the start of treatment or when increasing the dosage, with 25% and 10% of subjects reporting nausea and vomiting or diarrhoea, respectively (454, 455). Contrary to original postulations, recent studies report that these side effects occur even in fasting individuals, implying that they are not associated with GLP-1 GI functions and delayed gastric emptying, but rather with direct interactions with CNS GLP-1Rs, particularly in the brainstem's area postrema (454, 455).

Other factors, such as the specific type of GLP-1R agonists and pharmacogenomics, may explain variation in responses to GLP-1R agonists between individuals. For example, several clinical trials and meta-analyses demonstrated that long-acting GLP-1R agonists like liraglutide, dulaglutide, and once-weekly exenatide, had higher adherence rates and fewer adverse effects compared to short-acting agents (437, 444). Yet, liraglutide has also been reported to have the highest risk of pancreatitis (456) (452). As for pharmacogenomics, GWAS studies have identified genes including *Wfs-1*, *Cnr-1*, *Sorcs1*, *Kcnq1*, *Tcf7l2*, *Ctrb1/2* and *Glp1r*, to be involved in variation in GLP-1R agonist responses (40, 457-459). Further, a recent study investigated seven GLP-1R single nucleotide polymorphisms (SNPs) in 152 T2D patients. Results revealed associations between these variants and liraglutide induced GIARs, identifying three SNPs: rs2254336 (intron 3 variant, minor allele frequency (MAF)=55.75%), rs3675468 (Lys130Lys, MAF=20.63%), and rs3765467 (Arg131Gln, MAF=22.72%) (460).

These SNPs are not only linked to an increase in liraglutide-dependent GIARs, but are also in linkage disequilibrium, and tend to co-occur in an individual, potentially exacerbating adverse effects (293).

Given these points, clinicians should be vigilant about monitoring drug dosage and choice of GLP-1R agonist depending on the pharmacogenomic and health backgrounds of individuals, to ensure maximum responses along with safety and tolerability. Also, patients should be made aware of the mode of action of these drugs, mainly deceleration of gastric emptying, that could lead to nausea because of eating when already feeling satiated.

1.5.3 Incretin-based therapies: dual and triple incretin receptor agonists

The glucagon receptor family, comprising GLP-1R, GLP-2R, GIPR, and GCGR, shares sequence and structural similarities. They exert their effects through similar G protein mediated mechanisms, but they exhibit tissue-specific traits and vary in localisation across different tissues, accounting for their different functions and signalling modes. Receptor mediated multi-organ effects and evidence from gastric bypass procedures, which show simultaneous increases in the secretion of GLP-1 and other gut-derived hormones, suggest potential synergistic effects that could reduce adverse outcomes and increase drug tolerability (256, 461, 462). Unimolecular dual and triple agonists of GLP-1R, which activate GLP-1R alongside one (dual) or two additional receptors (GIP, GLP-2R or GCGR) (triple), are emerging as a new class of therapeutic agents for metabolic diseases like obesity and T2D (463). These chimeric dual or triple agonist peptides target multiple signalling pathways, both centrally and peripherally, to provide superior glycaemic control, appetite reduction and weight loss benefits compared to monotherapies, thereby improving treatment outcomes.

Interest in developing these agents was sparked by studies showing that combining GLP-1 signalling with GIP, glucagon (GCG), or both enhanced insulinotropic responses beyond what GLP-1 alone can achieve (464).

Dual agonists

The complementary actions of the incretins, GIP, and GLP-1, in response to food ingestion, including promotion of GSIS, inhibition of glucagon secretion, and reducing weight and appetite form the rationale behind the development of dual GLP-1R/GIPR agonists, also known as “tw incretins” (465). In addition to metabolic and non-metabolic effects, individual incretins like GLP-1 offer protective actions at the heart, liver, and renal levels. GIP exhibits anti-obesity effects, promotes fat deposition, and contributes to bone growth (256, 461). These factors suggest that GIPR/GLP-1R co-agonism could lead to improved outcomes in managing diabetes. Active GLP-1R/GIPR co-agonists were first described in a series of studies done between 2006 and 2013 in rodents, non-human primates, and humans (233). From several candidate molecules, a single peptide with balanced activity at both incretin receptors and minimal GCGR activity was chosen for further experimentation (233). This original GLP-1R/GIPR co-agonist was trialled in obese mice after some modifications, demonstrating greater body weight and food intake reductions, improved glycaemic control, and lipid outcomes compared to exendin-4 and liraglutide (233, 466). Although these results along with findings from phase I clinical trials were promising, phase II trials with a once-daily regimen by Novo Nordisk (NNC0090-2746), showed no significant improvement compared to liraglutide at the dose tested (466, 467). This along with concurrent studies in comparison to once-weekly GLP-1R agonists showed that these were still better than the GLP-1R/GIPR co-

agonist, resulting in abandonment of this compound in favour of incretin combination therapy (high-potency GIPR agonist + 2.4 mg semaglutide once weekly) (256).

Tirzepatide, another “tw incretin” agonist, had more clinical success and has been FDA approved for use in the management of T2D and obesity in 2022. Unlike the first GLP-1R/GIPR agonist developed, tirzepatide was developed by selective modifications of the native GIP sequence, rather than a glucagon/GLP-1 agonist, to achieve agonism at the GLP-1R (468). Pharmacological studies demonstrate that tirzepatide is an imbalanced, biased agonist at the GLP-1R, maintaining normal engagement at the human GIPR (469). The fatty acid moiety appears to be crucial in determining the agonist engagement, trafficking, and signalling of both GLP-1R and GIPR, as well as the pharmacokinetic profile (373, 469, 470). Although the fatty acyl group does not affect binding and signalling of GIPR, it potentiates biased agonism at the GLP-1R, favouring cAMP generation over β -arrestin recruitment, and retention at the cell membrane (373, 470). Clinical trials comparing tirzepatide with other once-weekly GLP-1R mono-agonists such as dulaglutide and semaglutide, showed impressively greater reductions in HbA1c, fasting glycemia, lipid levels, and weight loss after several weeks (471). Tirzepatide’s remarkable weight loss effects were further highlighted in clinical studies with obesity subjects only, where more than 50% of patients treated with 10-15 mg tirzepatide achieved over 20% weight reductions (472). Despite observation of meaningful reductions in blood pressure, clinical assessments of cardiovascular outcomes of tirzepatide are still ongoing (473).

Oxyntomodulin, a proglucagon derivative like GLP-1 and glucagon, acts as an endogenous dual agonist of GLP-1R and GCGR, possesses similar affinity and potency at both receptors, and has been shown to exert greater weight loss and equivalent glycaemic control compared

to GLP-1R agonists in rodents and humans (461, 474, 475). This, in addition to the fact that GCGR activation induces additional beneficial metabolic effects such as enhanced energy expenditure, stimulation of hepatic lipolysis, and inhibition of food intake centrally via activation of PKA/CaMKKb/AMPK dependent pathways in the ARC makes the GCGR an attractive partner for GLP-1R agonism (476, 477). As such, research into long-acting GCGR/GLP-1R dual agonists are garnering attention, with cotadutide (MEDI-0382, AstraZeneca), mazdutide (IBI362, Eli Lilly), and bamadutide (SAR425889), currently being trialled in the clinic (461, 463). Data from these clinical trials has generated guarded optimism over the effects on weight reduction (~10% reduction over 12 weeks) and energy expenditure increase in obese adults (478-480). Ultimately, further GLP-1R/GCGR compounds are in development, especially as GCGR activation not only reduces body weight but also stimulates hepatic lipolysis, thermogenesis, making a promising target for obesity and hepatic steatosis, despite the counterintuitive effects that the receptor might have increasing hepatic glucose outputs.

GLP-2, another incretin product of proglucagon secreted by intestinal L-cells, exhibits intestinotrophic effects, enhances gut barrier function, intestinal mucosal proliferation, reduces satiety and rates of gastric emptying (481). Thus, GLP-2R agonism provides additional protection from obesity-associated complications such as systemic low-grade inflammation via enhanced gut barrier function and non-alcoholic fatty liver disease (NAFLD) by regulating lipid homeostasis and downregulating cytokine expression in hepatic stellate cells (481, 482).

Dapaglutide, a first-in-class GLP-1R/GLP-2R agonist, demonstrates significantly enhanced oral glucose tolerance, reduced intestinal transit time, and intestinal growth in mouse models

of short bowel syndrome (483). Clinical studies in 40 healthy male subjects also showed dose-dependent weight loss after administration of dapaglutide once-weekly for 4 weeks (484). Therefore, GLP-1R/GLP-2R agonists represent a promising approach to treat gastrointestinal diseases, NAFLD, and obesity.

Triple agonists

Given the success of GLP-1R/GIPR and GLP-1R/GCGR agonists in the clinic, a novel approach has been developed to further leverage the synergy between the glucagon receptor family members. This involves generating single-molecule agonist peptides that exhibit triple activity at all three receptors (462, 463). Indeed, pre-clinical and clinical trials of SAR441255 (Sanofi), a GLP-1R/GIPR/GCGR triple agonist, demonstrated superior results and improved glycaemic control compared to dual GLP-1R/GCGR agonists (485). Likewise, two other triple agonists under development, NN9423 (Novo Nordisk) and retratrutide (LY3437943, Eli Lilly) (486, 487), have shown similar success in clinical trials. Obese patients administered retratrutide once-weekly experienced up to 24% weight loss over 11 months (486, 487). Additionally, NN9423 demonstrated superior potency and efficacy over semaglutide, tirzepatide, and other tri-agonists, without compromising glycaemic control in obese mouse models (462, 464). Moreover, tri-agonist studies suggest that optimal weight loss effects are achieved when the receptor potency ratio favours GCGR over the other receptors (462, 464). Therefore, triple agonists balanced at each receptor should be further explored for optimal clinical outcomes.

Overall, although the use of dual and tri-agonists is a popular area of investigation, the potential impact of biased signalling from each of the different receptors provides a further level of complexity that must be considered. Ideally, structural studies and clinical data from

patients with specific metabolic diseases should be integrated to correctly prescribe agonists, achieving optimal therapeutic benefits.

1.5.4 Non-Peptide agonists and allosteric modulators

Despite the immense success that injectable peptide GLP-1R agonists like semaglutide and tirzepatide have had in the clinic, there are still limitations with these peptide drugs including cost of production, adverse side effects, and low tolerability due to injectable route of administration (485, 487). Thus, highlighting the need for oral agonists which are more conveniently accepted, increasing compliance in patients, particularly when being taken with additional medications, as is often the case in the management of chronic metabolic diseases like diabetes and obesity. As mentioned before, prior efforts by developing an oral form of semaglutide were successful, yet this formulation also has limitations including constraints regarding administration and more severe GIARs than with injectable GLP-1R agonists. Therefore, there is significant competition and interest in the pharmaceutical field to develop oral small molecule non-peptide GLP-1R agonists, increasing bioavailability, convenience, compliance, and tolerability by diabetic patients who are most likely taking additional medications (485). Development of these small molecule agonists has proved challenging as few have been described for the class B GPCRs compared to class A and C. In fact, several allosteric agonists affecting receptor responses to natural ligands have been found for class A and C receptor families compared to class B.

Nevertheless, recently a diverse array of allosteric non-peptide ligands has been reported including quinoxalines, pyrimidine and pyrazolopyridine derivatives. Two compounds emerging from these studies have progressed to preclinical and clinical trials (485, 487).

Initial studies screening of 500,000 small molecule compounds by Novo Nordisk in 2006, identified a series of quinoxalines, of which compound 2 and later compound 9 showed allosteric GLP-1R activation potential, potentiating GSIS in mouse islets by increasing endogenous GLP-1 and oxyntomodulin affinity to the receptor (488). Interestingly, these compounds also exhibited GLP-1R specific activity and inhibition of antagonist [exendin-4 (9-39)] responses at the receptor, validating their allosteric mode of activation (488). Although these studies demonstrated beneficial effects of positive allosteric modulators (PAMs) on GLP-1R activity and insulintropic function, they exhibited poor chemical stability due to high microsomal metabolism, necessitating further work in this area to achieve small molecule compounds with more chemical stability whilst exploiting these allosteric binding sites (487).

Later in 2010, Eli Lilly identified a series of pyrimidine based positive ago-allosteric modulators of GLP-1R such as the racemic compound 13, otherwise known as BETP, by screening a small library of 3D-pharmacophore models in human GLP-1R expressing HEK293 cells (487, 489). Ago-allosteric modulators are defined as compounds that act as allosteric modulators and independent agonists at GPCRs, increasing the affinity of orthosteric ligands for the receptor (487). This compound exhibited GLP-1R dependent beneficial insulintropic effects by acting in an additive manner, including increasing GSIS in rodent and human islets, improving glucose tolerance *in vivo* with IPGTTs and hyperglycaemic clamp assays, and being resistant to antagonist action (489). Whilst its *in vivo* results and stability in plasma are encouraging, akin to the quinoxaline derivatives, they also need more work in terms of metabolic liability and chemical stability in the presence of nucleophiles before longer term studies are performed (487).

The first small molecule non-peptide GLP-1R agonist to advance to clinical trials, danuglipron (PF-06882961 or compound 6), is a fluoropyrimidine based compound, discovered by Pfizer as part of a BETP-sensitised high-throughput cAMP assay screen (490). Danuglipron possesses a pharmacological profile resembling GLP-1 more than other injectable GLP-1R agonists like exendin-4, liraglutide, and even oxyntomodulin. It fully activates cAMP signalling, but acts as a partial agonist of calcium mobilisation, pERK1/2, and β -arrestin recruitment (490). Pre-clinical studies in animal models demonstrated significantly increased insulin levels and reduced food intake in primates but not in rodents, later revealed to be due to the requirement of a primate specific tryptophan residue in the agonist binding pocket (490). Further clinical trials in humans, revealed dose dependent reductions in body weight, HbA1c, and fasting plasma glucose (FPG) (1.16% HbA1c, >30mg/dL FPG, >10% body weight) in T2D and obesity patients with twice-daily danuglipron administration (491, 492). Albeit, akin to injectable GLP-1R agonists, it is associated with high rates of adverse GIARs (73% nausea, 47% vomiting, 25% diarrhoea), and a greater than 50% discontinuation rate (493, 494). As a result, the twice-daily danuglipron has not advanced into phase III studies, and a once-daily formulation is being pharmacokinetically investigated in the hope of better tolerability outcomes.

Eli Lilly's orforglipron (LY3502970, OWL-833), another oral small molecule once-daily non-peptide and a pyrazolopyridine derivative identified in a screen of urokinase-type plasminogen activators, has had more success in clinical trials (495, 496). This compound is characterised by its biased activity towards cAMP signalling and distinct structural activation of the GLP-1R. Orforglipron binding to the GLP-1R results in a distinct ECD and TMD structural conformation as a result of differential interactions with residues within the ECD, TM1-3,

ECL2 and TM7 (310). Furthermore, it appears to be comparable with other injectable GLP-1R agonists and oral semaglutide in T2D management (497, 498). With a half-life of 29-49 hours, it has shown a continuous decrease in BMI, an 8.6% reduction in body weight, and clinically significant reduction in systolic blood pressure after 26 weeks of once-daily administration at a 12 mg dose (498). Orfoglipron is currently in phase III trials and offers an accessible and tolerable alternative to oral semaglutide, with similar weight management and glycaemic benefits (497, 499).

Given all the potential benefits of oral small molecule non-peptide and positive allosteric agonists of the GLP-1R, more studies are needed to understand the underlying differences in structural binding pockets, conformational activation and signalling pathways. Indeed, these differences are increasingly viewed as crucial for their biological understanding, including elucidating biological differences between small molecules and peptide agonists to develop therapeutics with greater efficacy (287). Alternatively, PAMs offer more potential undiscovered opportunities to develop effective GLP-1R agonists, additively potentiating endogenous GLP-1(7-36) and GLP-1(9-39) effects (309).

1.6 GPCR Pharmacogenomics: genomic variation in the human *Glp1r* gene

1.6.1 Pharmacogenomics of GPCRs

Pharmacogenomics is the study of how genetic variation affects an individual's response to drugs. It combines pharmacology, the science of drugs, and genomics, the study of genes and their functions, to develop precision medications, as in tailored medications and doses to an

individual's genomic profile. GPCRs are the most targeted proteins for the treatment of common diseases, mediating the effects of >100 of drugs in use in the clinic. Natural polymorphisms in the genes of targetable GPCRs can alter receptor structure, signalling and function, resulting in phenotypes such as association with disease related traits or pharmacogenetic traits. Thus, understanding genetic variation in druggable GPCRs has direct bearing on tailoring personalised healthcare and maximising clinical outcomes.

SNPs can cause changes in receptor structure, leading to opposing effects on receptor function and resulting in different phenotypes in individuals. These changes are classified into two categories: loss-of-function variants (LoF) and gain-of-function variants (GoF) (500). LoF variants are marked by a structural change in the receptor, resulting in a defective version that can affect the function of the normal receptor when expressed in heterozygous individuals (dominant-negative phenotype) (501). These LoF variants are further divided into five subtypes based on their impact on receptor function: defective receptor synthesis, improper trafficking to the cell surface and retention of the receptor in the ER, impaired binding to ligands and activation, defective receptor signalling via $G\alpha_s$ proteins, and mutants without known effects (501).

The phenotypes of LoF variants may be alleviated by the use of pharmacological chaperones that stabilise misfolded receptors allowing them to reach the cell membrane. On the other hand, GoF variants result in receptor structural changes that confer constitutive receptor activation with autonomous signalling and are thus also known as bearing constitutively activating mutations (CAMs) (502). Pharmacological interventions for GoF variants could include use of receptor antagonists to reduce signalling of the constitutive receptor. Therefore,

single nucleotide variants of GPCR genes should be classified based on their structural analysis, aiding in the prediction of phenotypic and pharmacogenetic traits.

A study utilising data from 2,504 healthy individuals from the 1000 Genomes Project, demonstrated that on average an individual has 68 missense variants within the coding region of about one-third of GPCR drug targets, of which about eight have been clinically associated with altered pharmacological responses and disease traits. For instance, the human dopamine D2 receptor (*D2r*) gene harbours several polymorphic variants, such as Ser311Cys (MAF=0.0225), which is associated with increased schizophrenia risk and response to antipsychotic drugs as a result of altered ability to form dimers with D1R (503). Similarly, GWAS studies have identified 18 genetic variants in the follicle stimulating hormone receptor (*Fshr*) gene which are significantly associated with polycystic ovary syndrome (PCOS) (504). Some FSHR variants were also associated with lower sensitivity to follicle stimulating hormone (FSH) therapy in the ovaries and lower oestradiol levels, meaning patients required higher FSH doses (505).

1.6.2 Class B GPCR genomic variants: Implications for health and therapeutics

Genes encoding members of the class B1 GPCR family also harbour missense variants. In fact, the calcitonin receptor (*Calcr*) has missense variants in more than 40% of its known functional sites, hence increasing the likelihood of variable drug responses amongst individuals (506). A screen of human *Calcr* polymorphisms identified 11 variants in the gene including the Pro447Leu (rs1801197) variant associated with osteoporosis and increased fracture risk in postmenopausal women (507). Similarly, another variant was identified that was associated with not just increased fracture risk but also higher serum androstenedione

levels in post-menopausal women (508). A recent study using aggregated natural genetic variant data for all class B1 GPCRs from genome aggregation database (gnomAD) revealed that the glucagon receptor (*Gcgr*), the *Glp1r*, the corticotropin-releasing hormone receptor 1 (*Crhr1*), and the calcitonin receptor-like receptor are under strong evolutionary selection against missense variations (negative selection), displaying the least missense variants (509). This study also mapped the mutational landscape of GCGR, identifying 250 missense variants across its sequence, including the disease association mutations, G40^{ECD}S associated with diabetes, P86^{ECD}S and V368M associated with Mahvash disease (hyperglucagonemia, α -cell hyperplasia and islet cell tumour) (509, 510). Structurally the V368M mutation is located at an important site for ligand and ECD recognition, the beginning of ECL3) (510). Subsequently, this variant also exhibited impaired, lower ligand binding affinity, $G\alpha_s$ recruitment and cAMP signalling in response to glucagon, compared to the wild-type receptor (509, 511). Despite the molecularly impaired receptor, the genetically engineered mouse model showed improved glucose tolerance, lower fasting blood glucose levels and adiposity (511). Interestingly, a recent study investigating missense variants using data from the UK Biobank, revealed that cAMP LoF missense variants, including G40S previously linked to non-insulin-dependent diabetes, are not necessarily linked to T2D, but to increased plasma proglucagon levels predisposing to T2D and obesity (512). Frameshift mutations in the *Gcgr* were associated with higher liver fat levels compared to wildtype individuals, which were also not observed in the cAMP LoF variants (512). This study highlights the need for further *in silico* studies and experiments to pharmacologically characterise the *Gcgr* variants, and understand the multiple factors impacted by receptor signalling such as regulation of the proglucagon levels.

Gipr is another member of glucagon receptor family that also harbours 597 missense variants according to gnomAD, as well as possessing 13 different splice variants with uncertain functions. Moreover, the GIPR missense variant, rs1800437 (E354Q), has been reported to reduce GIPR signalling by increasing receptor desensitisation and downregulation, and is associated with higher BMI, 2-hr glucose levels and T2D risk (513). On the other hand, another study showed that two other rare missense *Gipr* variants rs139215588 (R190Q) and rs143430880 (E288G), despite displaying reduced G protein coupling, as well as impaired β -arrestin recruitment and internalisation, were associated with lower BMI in carriers (514). Thus, these findings suggest that reduced GIPR signalling can have both beneficial and disadvantageous effects on human physiology and that *Gipr* missense variants need to be explored further for a more complex picture of the GIPR signalling to exploit its therapeutic potential. Splice variants (SVs) also add another layer of complexity to GIPR mediated signalling and function. Two C terminus-truncated GIPR SVs have been investigated so far, GIPR-201 and GIPR-203 (515, 516). GIPR-201 is a 419-residue polypeptide that coexists with the wildtype receptor and differentially affects its signalling profile (515). GIPR-203, a smaller SV consisting of 265 residues, is localised to β -cells where it reduces receptor intracellular trafficking and expression of the wild-type receptor in high-fat diet fed mice, suggesting an association with obesity (516). SVs were also found to be differentially modulated by the action of RAMPs compared to the untruncated protein, suggesting more complex GIPR modulation mechanisms that require further investigation to develop improved therapeutics (517).

1.6.3 Overview of GLP-1R variants: altered receptor function, agonist response, and associated traits

Pharmacogenetics is the study of how inter-individual genetic variations influence individual responses to therapeutic drugs. Through this field, scientists aim to develop effective, safe medications and doses tailored to a person's genetic makeup, known as "precision medicine". Several clinical studies have observed that while GLP-1R agonists are a highly effective class of T2D therapeutics, some individuals respond better than others, and some may experience more adverse effects compared to others (293). Although this could be attributable to factors such as patient adherence and distinct circulating drug levels, genomic variants in the *Glp1r* loci may be linked to therapeutic responses in a mechanistically resolvable manner (293). Additionally, studies of GLP-1R variants can have a significant impact on therapeutic development as they reveal residues important for ligand binding, signalling, and trafficking of the receptor, as well as alternative receptor activation mechanisms. A growing body of research has documented variations in the *Glp1r* loci, including nonsynonymous SNPs that alter GLP-1 binding affinity, intracellular signalling, glycaemic response, weight loss, T2D and cardiovascular risk (43, 44, 518) (Table 1.2). An early study by Tokuyama *et al.* (2004) identified five missense variants in the *Glp1r* loci in a Japanese diabetic and non-diabetic cohort. Amongst those, the T149M mutation was detected in diabetic patients and was found to be associated with defective glycaemic response, insulin secretion and sensitivity (519). These findings are supported by further studies assessing this variant in COS-9 and HEK293 cells, where it exhibits reduced agonist-binding affinity, and reduced recruitment of mini-G_s and β -arrestin 2, thus resulting in loss of function for cAMP, pERK1/2, and calcium signalling pathways (382, 520). Similarly, insights from molecular dynamic simulations examining the

full length active T149M structure bound to GLP-1 and $G\alpha_s$ protein, showed that as the variant is localised in the vicinity of the ligand binding pocket, the substitution results in a change in the TM1 tilt and ICL1 conformation, displacing residues involved in coupling to the $G\alpha_s$ and β -arrestin 2 proteins (521).

Data from genome wide association studies this past decade, have revealed novel *Glp1r* coding variants associated with glycaemic traits such as fasting glucose, random glucose and T2D risk (43, 518, 522). A notable example is the common *Glp1r* missense variant G168S (rs6923761, MAF=0.2189), which showed an association with small reductions in blood glucose levels, increased GLP-1R internalisation and mini- G_s recruitment compared to the wild-type receptor when stimulated by GLP-1 (518). Physiologically carriers of this mutation exhibited lower glucose, BMI, triglyceride, and leptin levels (523). It has also been associated with differences in pharmacological responses to incretin-based diabetes therapies. Whereby diabetic patients expressing the variant exhibit greater weight loss on liraglutide (524), but an attenuated glucose lowering and insulin secretion effect in response to DPP-4 inhibitors and GLP-1 infusion, respectively (525, 526).

Table 1.2 List of all human *Glp1r* SNPs Details include their coding consequences, allele frequencies, and associated clinical outcomes

Variant ID	Coding Consequence	Allele Frequency	Associated Clinical Outcome	Reference for Clinical Associations
rs10305420	P7L	0.275335061	Impaired β cell insulin secretion and β cell survival, Reduced response to exenatide Decreased glycated haemoglobin levels, Heart failure, Poor response and weight loss in response to liraglutide, NAFLD in the SHARE Scottish medical records	Yu, Wang, Liu and Cao, 2019 Daghlis et al., 2021 Jensterle et al., 2015 B. McKinstry , et al. 2017
rs2295006	R44H	0.001669647	Bone Mineral Density in postmenopausal women, Glycaemic traits	O, Fedoryak, 2017
rs150253529	R48H	2.62892E-05	Colorectal cancer	Santarius et al., 2010
rs774357734	D67N	2.3903E-05	Associated with Random Glucose	Lagou et al., 2021
rs3765467	R131Q	0.016058001	Blood sugar levels	Qui X et al.,2020
			Increased Type 2 diabetes,	Zhang et al., 2020
			Increased risk of Parkinson's Disease, Male lean, and adipose tissue mutations	Li W et al., 2020
			Bone Mineral Density and Osteoporosis, Impaired β cell insulin secretion and Increased β cell apoptosis	Zeng Z et al.,2020
			Response to DPP4 inhibitors in T2D patients	Nishiya Y et al., 2020
			Nutrient-dependant decrease in insulin secretion	Dawed AY et al. 2016
			Decreased standard deviation of plasma glucose after exenatide 5ug treatment twice daily	C.H. Lin et al., 2015
			Better responses to liraglutide	Jensterle et al., 2015
rs147627784	G132R	6.57082E-06	Exogenous GLP-1 responses	Sathananthan A et al. 2010
rs112198	T149M	1.50E-05	Non-significant decrease in fasting glucose	Wessel et al., 2015
rs6923761	G168S	0.21897527	Higher Type 2 Diabetes risk, found in patient with T2D, impairs insulin secretory response to GLP-1.	D de Luis., et al, 2014
			Smaller HbA1c reduction after gliptin therapy	Zeng Z et al.,2020
			Higher alcohol consumption in humans	Suchankova et al., 2015
			Increased weight loss and metabolic improvement in diabetic patients treated with liraglutide	D de Luis., et al, 2015
			Decreased insulin secretion in non-diabetic patients after a 2h GLP-1 infusion	Sathananthan A et al. 2010
rs146340667	V194I	0.000282553	Less weight loss after Biliopancreatic diversion surgery	D de Luis, D Pacheco, R Aller, O Izaola, 2014
rs140642887	A239T	0.000368373	Associated with Random Glucose	Lagou et al., 2021
rs185053350	S261A	6.57168E-06	Associated with Random Glucose	Lagou et al., 2021
rs145619754	L268F	0.000151127	Associated with Random Glucose	Lagou et al., 2021
rs10305492	A316T	0.010692551	Non-significant reduction in fasting glucose	Wessel et al., 2015
			Impaired β cell insulin secretion and Increased β cell apoptosis, Reduced Fasting Glucose	Li W et al., 2020
			Reduced Type 2 Diabetes Risk	Wessel et al., 2015
			Early insulin secretion	Mahajan A. et al., 2015
			High 2-h glucose	Chen J et al., 2021
			Decreased HA1c levels	Scott et al.,2016
rs10305493	S333C	4.60115E-05	Reduced Cardiovascular Risk	Yapici-Eser et al., 2020
rs202171972	D344E	8.54061E-05	Reward Learning and Anhedonia	
rs527991362	A375T	1.97163E-05	lower T2D risk, lower fasting and random glucose, lower HbA1c	
			Associated with Random Glucose	Lagou et al., 2021
			Associated with Random Glucose	Lagou et al., 2021

1.6.4 The rs10305492 human *Glp1r* variant: a low-frequency missense variant associated with protection from T2D and cardiovascular disease

In the same vein, the same studies identified another GoF *Glp1r* low-frequency missense variant A316T (rs10305492, MAF=0.01069) associated with lower fasting and random glucose levels, altered insulin responses, lower T2D and cardiovascular risk (43, 518, 522). In a major recent paper investigating coding *Glp1r* variants, Lagou *et al.* 2023, further investigates the structural, pharmacological, as well as physiological effects of this variant by leveraging population data from the UK biobank in concert with *in vitro* and *in silico* experimental approaches (518). MD simulations modelling the oxyntomodulin activated A316T GLP-1R- $G\alpha_s$ complex showed that the variant induced residue-residue interactions within the central hydrogen bonding network, altering TM5/6 orientation and ICL3 conformational changes involved in G protein coupling. Consistent with this, complementation assays showed improved mini- G_s recruitment in response to GLP-1, semaglutide and tirzepatide but not to exendin-4 in HEK-293T cells expressing the A316T variant compared to the wild-type control (518).

In contrast to the opinion that increased signalling is often mirrored by increased surface expression and reduced internalisation, A316T exhibits around 50% reduction in plasma membrane localisation, and increased endocytosis in response to the GLP-1R agonists mentioned above (382, 527). Despite this defect in cell surface expression, parallel to previous findings, Gao *et al.* (2023) found that the A316T GLP-1R exhibits GoF for Ca^{2+} mobilisation and cAMP production in response to exendin-4 in HEK 293T cells (527). Interestingly, this variant also exhibited reduced β -arrestin 2 recruitment and ERK1/2 activation (527). However, in previous studies Koole *et al.* presented a contradicting loss in intracellular Ca^{2+} mobilisation

in response to the agonists GLP-1 and exendin-4 relative to the wild-type receptor in CHO cells (382). This difference in results could be attributed to use of different cell types to characterise calcium signalling in the variant.

So far, *in vitro* experiments have used heterologous expression systems, mainly CHO and HEK 293T cells, to examine GLP-1R variant effects, which as observed in previous studies results in incongruous findings. Therefore, it is crucial to characterise these variants, including A316T, at endogenous levels in their native cellular environment and *in vivo*.

Allele frequencies of the rs10305492 human Glp1r variant by ethnicity and sex

The allele frequencies of the rs10305492 *Glp1r* variant have been studied across different ethnicities and sexes, providing valuable insights into the distribution of this genetic variant. Based on data from the gnomAD database (528), the allele frequencies of the rs10305492 *Glp1r* variant (A/G) were examined across different ethnicities and sexes. Below is a detailed table summarising these allele frequencies:

Table 1.3 Heterozygote and Homozygote Frequencies for the rs10305492 *Glp1r* variant by ethnicity and sex.
Details include total allele count, allele number, homozygote and heterozygote count, and allele frequencies.

<i>Ethnicity</i>	<i>Sex</i>	<i>Total Allele Count</i>	<i>Allele Number</i>	<i>Homozygotes</i>	<i>Heterozygotes</i>	<i>Homozygote Frequency</i>	<i>Heterozygote Frequency</i>
<i>European (non-Finnish)</i>	XX	9880	611512	91	4900	0.000149	0.00801
	XY	9022	568296	79	4486	0.000139	0.00856
<i>European (Finnish)</i>	XX	435	30222	3	429	9.90E-05	0.0142
	XY	420	33812	0	420	0	0.01242
<i>Ashkenazi Jewish</i>	XX	166	14502	1	165	6.90E-05	0.01138
	XY	185	15102	0	185	0	0.01225
<i>Remaining</i>	XX	376	32846	3	373	9.10E-05	0.01135
	XY	356	29662	2	354	6.70E-05	0.01194
<i>Admixed American</i>	XX	251	32466	2	249	6.20E-05	0.00767
	XY	244	27542	4	240	0.000145	0.00871
<i>African/African American</i>	XX	118	41492	2	116	4.80E-05	0.0028
	XY	77	33548	0	77	0	0.0023
<i>Middle Eastern</i>	XX	9	2650	1	7	0.000377	0.00264
	XY	6	3412	0	6	0	0.00176
<i>Amish</i>	XX	1	470	0	1	0	0.00213
	XY	0	442	0	0	0	0
<i>South Asian</i>	XX	17	23186	0	17	0	0.00073
	XY	74	67886	2	72	2.90E-05	0.00106
<i>East Asian</i>	XX	1	22978	0	1	0	4.00E-05
	XY	1	21900	0	1	0	5.00E-05
<i>Total</i>	XX	11254	812324	103	5545	0.000127	0.00683
	XY	10385	801602	87	5479	0.000108	0.00684
<i>Combined</i>	Total	21639	1613926	190	11024	0.000118	0.00683

This table provides an accurate representation of the heterozygote and homozygote frequencies by ethnicity and sex based on the collated data from the gnomAD database and allele count definitions. Terms used in the above table are defined below:

Total allele count: the number of observations of the alternate allele across all chromosomes of the sample for example homozygous is two alleles per individual and heterozygous is one allele per individual.

Allele number: the total number of alleles samples.

Homozygotes: number of individuals homozygous for the minor (A) allele, each contributing two to the allele count.

Heterozygotes: number of individuals heterozygous calculated as half the total allele count minus twice the number of homozygotes (since each homozygote has two minor alleles).

Homozygote Frequency: Number of homozygotes divided by the total number of individuals.

Heterozygote Frequency: Number of heterozygotes divided by the total number of individuals.

Overall, this data suggests that there are no differences in the allele frequencies of rs10305492 between males and females within the same ethnic groups (Table 1.3). Despite slightly higher values for females compared to males, the distribution tends to be similar across sexes, indicating that sex does not play a major role in the variability of this allele. However, as previously mentioned in GWAS studies, this allele is most frequent in non-Finnish European populations and not present at all in Asian populations (43, 522) (Table 1.3). These differences

highlight the importance of considering genetic background in the study of GLP-1R related therapies and their metabolic effects.

Physiological and hormonal effects of the rs10305492 Glp1r variant before and after interventions influencing blood glucose levels in human participants

Recent studies by Dorsey-Trevino *et al.* 2022, investigated human participants free of disease that possessed the rs10305492 *Glp1r* variant, as well as leveraging data from the Study to Understand the Genetics of Acute Responses to Metformin and Glipizide in Humans (SUGAR-MGH) to understand the role of genetic variation at the *Glp1r* in the incretin response as well as the physiological and hormonal effects of polymorphisms such as the rs10305492 before and after glipizide and metformin administration (529). Therefore, in addition to utilizing data from the gnomAD database, I sought to obtain further detailed information on the rs10305492 variant in humans from the study by Dorsey-Trevino *et al.* (2022). To acquire this data, I reached out to Dr. Aaron Leong, the corresponding author of the paper, via email. In the email, I requested access to the detailed incretin response from human patients that are carriers of the minor allele of the rs10305492 variant or not, including plasma glucose, insulin, GLP-1 and GIP levels during an OGTT, which was necessary to enhance my understanding of this variant in humans. Data on the polymorphism included human participants that were carriers of the variant, heterozygotes with the (A/G) genotype, while wild type was defined as (G/G) genotype. Dr. Leong responded positively, and the requested data were provided. This additional dataset was integrated into my thesis as preliminary data, allowing for a more comprehensive analysis.

Supplementary Figure 1 shows the comparison of various glycaemic traits including glucose and insulin as well as plasma hormone levels between individuals with the G/G genotype and

those with the heterozygote A/G genotype of the rs10305492 variant. Despite findings in previous papers establishing a link between the minor A allele of the rs10305492 variant and lower random and fasting glucose levels. Glycaemic traits including plasma glucose and insulin levels assessed in the data below showed no significant difference between the two genotypes. Congruently, incretin plasma hormone levels were also not unaltered between the different genotypes. Therefore, these results suggest that the A/G genotype does not significantly affect these metabolic and incretin parameters in healthy human participants. The discrepancy between previous GWAS findings and this study may be due to differences in study design, population characteristics, or environmental factors. For example, previous studies are likely to have included a broader range of participants and a larger sample size, including homozygote individuals with two minor alleles rather than just heterozygotes and individuals with varying degrees of glucose tolerance, thereby capturing a more pronounced effect of the variant on glucose metabolism.

The SUGAR-MGH study included a diverse cohort of only 1,000 participants including participants from multiple ethnic backgrounds and comprised of individuals at high risk for T2D, specifically targeting those with metabolic syndrome, obesity or a family history of the disease. Yet, this study excluded individuals with overt diabetes, and those on medications affecting glycaemic parameters that could confound the pharmacological responses to glipizide or metformin. On the other hand, the Lagou *et al.* GWAS study was conducted on 476,326 individuals without diabetes, including participants from diverse ancestries with the majority being European (459,772) and the rest belonging to other ethnic backgrounds, to investigate the genetic determinants of random glucose levels. These methodological differences in participant selection likely contributed to the variation in findings between the

studies, particularly regarding the impact of the rs10305492 variant on glucose and insulin levels.

1.7 Thesis Aims and Objectives

Given that the rs10305492 (A316T) *Glp1r* naturally occurring missense variant is associated with insulin secretion from pancreatic β -cells at low ambient glucose, lower fasting glucose levels and reduced T2D risk, we hypothesise that the A316T phenotype can be explained by variations in the structure of the receptor giving rise to differences in ligand binding, trafficking, and/or signalling, and subsequently beneficial physiological effects in glucose homeostasis.

Accordingly, the purpose of this thesis is to characterise the A316T mutation and its mechanism of action, including molecular, phenotypic, and pharmacological alterations, in cell-based, *ex vivo* and *in vivo* models. The studies described in this thesis will be presented across four chapters with four specific aims:

Chapter III: To characterise the structural changes underlying the altered function of the A316T GLP-1R using resolved cryo-EM structures of the human GLP-1: GLP-1R (A316T): $G\alpha_s$ complex and molecular dynamics (MD) simulations.

Chapter IV: To characterise the A316T GLP-1R phenotype in rodent and human cell lines compared to the wild-type receptor, elucidating alterations in ligand binding, signalling and trafficking mechanisms.

Chapter V: To investigate the metabolic phenotypes, including pharmacological responses to GLP-1R agonists *in vivo*, of the A316T variant in genetically engineering humanised GLP-1R mice expressing the A316T mutation.

Chapter VI: To study the influence of the A316T variant in murine and human islets, examining whether the *in vitro* mutant profile translates into multi-cellular primary islets.

Chapter 2: Materials and Methods

2.1 Peptides, Plasmids, and Reagents

2.1.1 Peptides

The endogenous GLP-1R agonist GLP-1(7-36)NH₂ was purchased from Bachem, Switzerland. exendin-4, exendin-F1 and exendin-D3 were all custom synthesized by Wuxi Apptec/Insight Biotechnology, China and were at least 95% pure. The long-acting agonists semaglutide and tirzepatide were obtained from Imperial College London Healthcare NHS Trust pharmacy.

2.1.2 Reagents

Tetramethylrhodamine-labelled exendin-9 and exendin-4 (exendin-9-TMR and exendin-4-TMR) have been described and validated before (390, 530). Exendin-9-Cy5 (LUXendin645) has been described before (228) and is a kind gift from Prof David Hodson (Oxford Centre for Diabetes, Endocrinology and Metabolism, UK). SNAP-Surface fluorescent probes were purchased from New England Biolabs. Surface cleavable BG-S-S-649 (364, 531, 532) was a gift of Dr Ivan Corrêa Jr, New England Biolabs.

2.1.3 Plasmids

The pSNAP/FLAG-tagged human *Glp1r* (*hGlp1r*)^{A316T} and *hGlp1r*^{A316T}-SmBiT constructs were generated *in house* by site-directed mutagenesis from pSNAP/FLAG-*hGlp1r*^{WT} (Revvity) and *hGlp1r*^{WT}-SmBiT (362) with the following primers:

Forward: 5'-CCGGCTGCCCATTCTCTTTACCATTGGGG-3', and Reverse: 5'-ACCCCAATGGTAAAGAGAATGGGCAGCCG-3' for pSNAP/FLAG-*hGlp1r*^{A316T}; and Forward: 5'-CCGGCTGCCCATTCTCTTTACCATTGGGG-3', and Reverse: 5'-ACCCCAATGGTAAAGAGAATGGGCAGCCG-3' for *hGlp1r*^{A316T}-SmBiT, using the QuikChange Site-Directed Mutagenesis Kit (Agilent) following the manufacturer's instructions.

Table 2.1 List and origin of plasmids used during this PhD project.

Name	Origin	Assay
<i>hGlp1r</i> ^{WT} -SmBiT (362)	Generated in house	NanoBiT complementation
<i>hGlp1r</i> ^{A316T} -SmBiT	Generated in house	NanoBiT complementation
SNAP/FLAG- <i>hGlp1r</i> ^{WT}	Cisbio	Nb37 bystander assay
SNAP/FLAG- <i>hGlp1r</i> ^{A316T}	Generated in house	Nb37 bystander assay
mini-G _s - LgBiT (533)	A gift from Prof Nevin Lambert (Medical College of Georgia, USA)	NanoBiT complementation
mini-G _{i/o} - LgBiT(533)	A gift from Prof Nevin Lambert (Medical College of Georgia, USA)	NanoBiT complementation
β-arrestin 2-LgBiT (362, 534)	Promega, UK, #CS1603B118	NanoBiT complementation
Gα _s (human, short isoform)	a gift from A. Inoue, Tohoku University, Japan	Nb37 bystander assay
Gβ1 (human)	a gift from A. Inoue, Tohoku University, Japan	Nb37 bystander assay
Gγ2 (human)	a gift from A. Inoue, Tohoku University, Japan	Nb37 bystander assay
RIC8B	a gift from A. Inoue, Tohoku University, Japan	Nb37 bystander assay
Endofin-LgBiT	a gift from A. Inoue, Tohoku University, Japan	Nb37 bystander assay
CAAX-LgBiT	a gift from A. Inoue, Tohoku University, Japan	Nb37 bystander assay

SmBiT- Nb37	a gift from A. Inoue, Tohoku University, Japan	Nb37 bystander assay
Endo (2xIFYVE)- ^T EPAC ^{VV}	Generated in house	cAMP assay in INS-832/3 cells
^T EPAC ^{VV}	A gift from K. Jalink, the Netherlands Cancer Institute, Netherlands	cAMP assay in INS-832/3 cells
pcDNA3.1	Addgene	Control plasmid

2.2 *In vitro* experiments

2.2.1 Mammalian cell culture

All INS-1 832/3 (rat insulinoma cell line) derived cells, were cultured in RPMI-1640 medium (Invitrogen), supplemented with 10% FBS, 100U/ml penicillin, 0.1 mg/ml streptomycin, 1 mM sodium pyruvate, 10 mM HEPES and 50 μ M 2-mercaptoethanol at 37 °C with 95 % O₂/ 5 % CO₂. The stable SNAP-GLP-1R expressing INS-1 832/3 cells (wild-type (WT) and A316T variant), derived from the parental INS-1 832/3 *Glp1r*^{-/-} cells, were generated in our laboratory by plasmid transfection, G418 selection, followed by FACS of the SNAP-GLP-1R-positive cell population. The pSNAP/FLAG-*hGlp1r*^{A316T} plasmid was generated in house from pSNAP/FLAG-*hGlp1r*^{WT} (Cisbio) by site-directed mutagenesis as described in section 2.1.3. These cells were maintained in the same medium mentioned above, with the addition of 1 mg/mL G418. Parental rat INS-1 832/3 cells with endogenous GLP-1R eliminated by CRISPR/Cas9 (535) (INS-1 832/3 *Glp1r*^{-/-}) were a gift from Dr. Jacqui Naylor, AstraZeneca.

EndoC- β H3 (immortalised human pancreatic β -cell line) were cultured in Advanced DMEM/F-12 (Thermo Fisher Scientific), 6.7 ng/ml sodium selenite, 10 mM nicotinamide, 5.5 μ g/ml human transferrin, 2 mM L-glutamine, 2% bovine serum albumin (BSA) Fraction V

(Roche), 1% P/S solution and 50 μ M β -mercaptoethanol. Cells were seeded in flasks or dishes coated with 2 μ g/ml fibronectin and 1% extracellular matrix (ECM) (#E1270, Sigma-Aldrich) and DMEM containing 100 μ g/ml streptomycin (P/S).

2.2.2 Fluorescent labelling of SNAP-hGLP-1R cells for confocal microscopy

INS-1 832/3 *Glp1r*^{-/-} cells transiently or stably expressing the SNAP/FLAG-hGLP-1R^{WT} or SNAP/FLAG-hGLP-1R^{A316T} (herein referred to as SNAP-hGLP-1R^{WT} or SNAP-hGLP-1R^{A316T}) were seeded onto 13-mm clean glass coverslips at a density of 5 x 10⁴ cells/well in 24-well plates and incubated overnight at 37°C in 5% CO₂, washed twice in 1x PBS and fixed with 4% paraformaldehyde (PFA) at 4°C for 20 min, then mounted onto slides in Diamond Prolong anti-fade with DAPI (ThermoFisher Scientific). Cell surface receptor expression was determined via fluorescent imaging using a Zeiss LSM-780 inverted confocal laser scanning microscope using 20x air objective (DIC Plan Apochromat 20x/0.8air, working distance 0.55mm). Acquired fluorescent intensity was quantified using Fiji (v1.53a).

2.2.3 Inhibition of lysosomal targeting in cells

INS-1 832/3 SNAP-hGLP-1R^{WT} and SNAP-hGLP-1R^{A316T} cells were plated on coverslips and incubated for 2 hours in complete medium with or without (100 nmol/l, or 400 μ M) bafilomycin-A1 (Sigma-Aldrich, UK), an established vacuolar H⁺-ATPase trafficking inhibitor (536). Cells were then washed with PBS and labelled with 1 μ M SNAP-Surface 549 (New England Biolabs) in complete medium at 37 °C for 10 minutes. Medium was then removed, cells were washed with PBS and fixed in 4% PFA, mounted in Prolong Diamond

antifade reagent with DAPI. Images were acquired by confocal microscopy with an Imperial College London NHI FILM Zeiss LSM-780 inverted confocal laser-scanning microscope and a 63×/1.4 numerical aperture oil immersion objective. Images were analysed using Fiji v1.54i software.

2.2.4 High content confocal microscopy assays for trafficking

Internalisation assays

INS-1 832/3 SNAP-hGLP-1R^{WT} and SNAP-hGLP-1R^{A316T} cells were seeded into poly-D-lysine-coated black 96-well plates for 24 hours before the assay. Labelling was then performed for 30 minutes with cleavable BG-SS-649 (1μM, a gift from New England Biolabs) at 37°C, a surface-labelling SNAP-tag probe that can be released on application of reducing agents such as MesNa. Cells were then washed once with HBSS before application of agonist or vehicle treatments for either 1 hour, 30 min, 15 min, or 5 min, at 37°C in complete medium. Agonist was removed by washing cells with cold HBSS, and subsequent steps were carried out at 4°C. MesNa [100 mM in alkaline TNE buffer (20 mM TrisHCl, 1 mM EDTA, 150 mM NaCl, pH 8.6)] or alkaline TNE buffer without MesNa was applied for 5 min and then washed with HBSS. Microplates were imaged immediately without fixation, using a modular microscope platform from Cairn Research incorporating a Nikon Ti2E, LED light source (CoolLED), fitted with a 20x phase contrast objective, assisted by custom-written high content analysis software implemented in Micro-Manager. A minimum of 9 images per well was acquired for both epifluorescence and transmitted phase contrast. Internalised GLP-1R, indicated by cell-associated fluorescence intensity, was analysed by flat-field correction of fluorescent images using the BaSiC plugin (537), segmentation of cell-containing regions from phase contrast

images using Phantast (538), and then fluorescence intensity was quantified for cell containing regions. The percentage of internalised receptor was calculated as follows:

Equation (1)

$$\frac{(F_{+Me}(t_x)/(F_{-Me}(t_x)) - (F_{+Me}(t_0)/(F_{-Me}(t_0)))}{1 - (F_{+Me}(t_0)/(F_{-Me}(t_0)))}$$

Where $F_{+Me}(t)$ and $F_{-Me}(t)$ are mean fluorescence $\pm$ MesNa at time t_x (5, 15, 30, or 60 min) or t_0 (0 min). Percentages were then plotted to calculate the area under the curve (AUC).

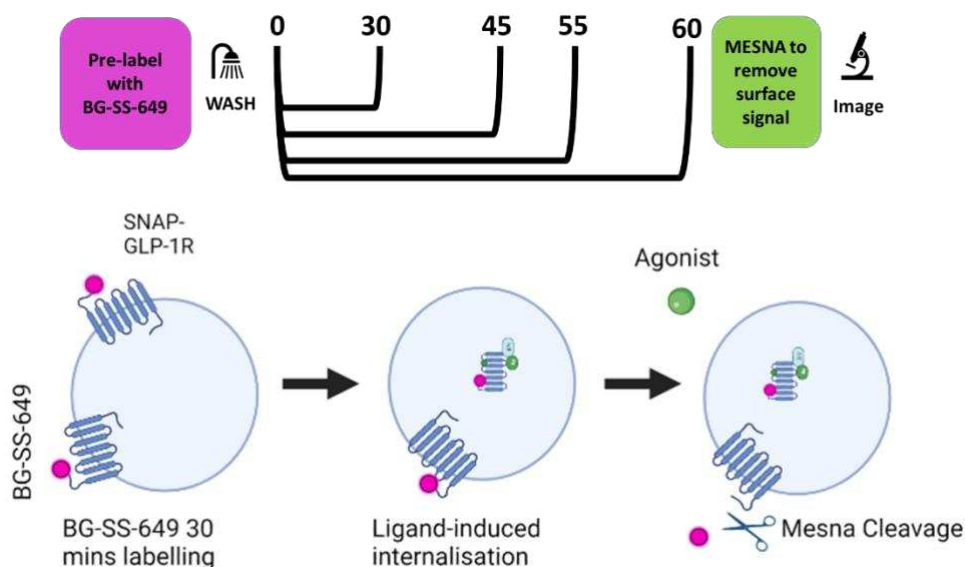


Figure 2.1 Scheme representing the MesNa based internalisation assay in INS-1 832/3 SNAP-hGLP-1R^{WT} or hGLP-1R^{A316T} cells

Recycling assays

INS-1 832/3 SNAP-hGLP-1R^{WT} and SNAP-hGLP-1R^{A316T} cells were seeded as above. After 24 hours, cells were washed with HBSS and pre-treated with vehicle or agonist for 1 hour at 37°C in complete INS-1 832/3 culture medium. Cells were subsequently washed to remove

agonists and treated with exendin-4-TMR (100 nM) in reverse time order for 3 hours and 1 hour at 37°C in complete medium. The microplate is promptly imaged after washing in the same modular system as described for the internalisation assay above. In this assay, total uptake of exendin-4-TMR is indicative of residual surface receptor at the end of agonist pre-treatment and cumulative reappearance of the surface receptor during the recycling period.

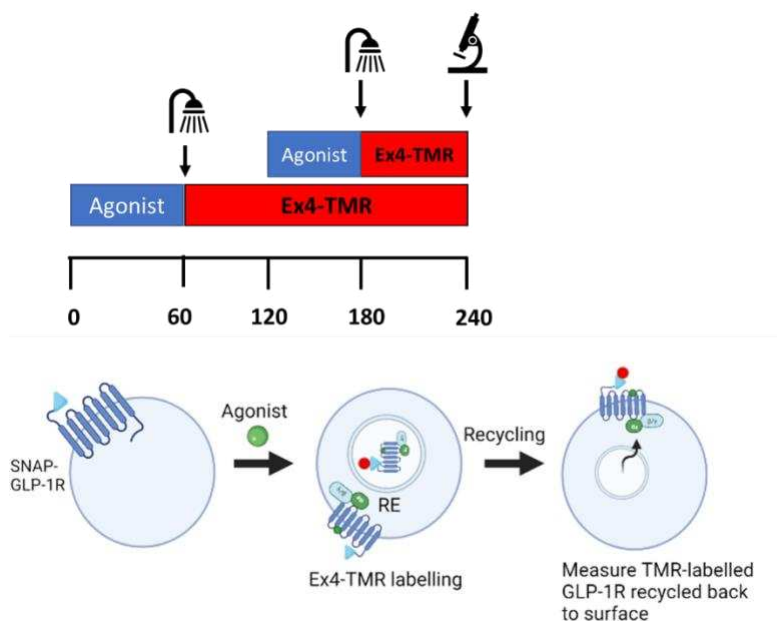


Figure 2.2 Scheme representing the recycling assay using Exendin-4TMR in INS-1 832/3 SNAP-hGLP-1R^{WT} or hGLP-1R^{A316T} cells

Degradation assays

INS-1 832/3 SNAP-hGLP-1R^{WT} and SNAP-hGLP-1R^{A316T} cells were seeded as above for internalisation and recycling assay. Medium was removed, and cells were washed with HBSS before addition of 50 µg/ml cycloheximide in serum-free medium (SFM), halting protein translation. After one hour, a time course was performed with the agonist, added in reverse time order, starting with 8 hours/4 hours/2 hours/1 hours/30 minutes /0 minutes. For the final 30 minutes of the experiment, the medium is replaced with 1 µM BG-OG (SNAP-labelling

fluorescent probe) in complete medium, to label total residual GLP-1R levels. Cells were then washed three times with HBSS prior to imaging the microplate as described for the internalisation assay, using a green fluorescence filter set. Total GLP-1R was quantified as the average cellular fluorescence labelling per well (as defined above for the Internalization assay), with further subtraction of non-specific fluorescence (i.e. INS-1 SNAP-hGLP-1R cells without BG-OG labelling). Results were expressed as a percentage relative to labelled cells without agonist treatment and AUCs recorded.

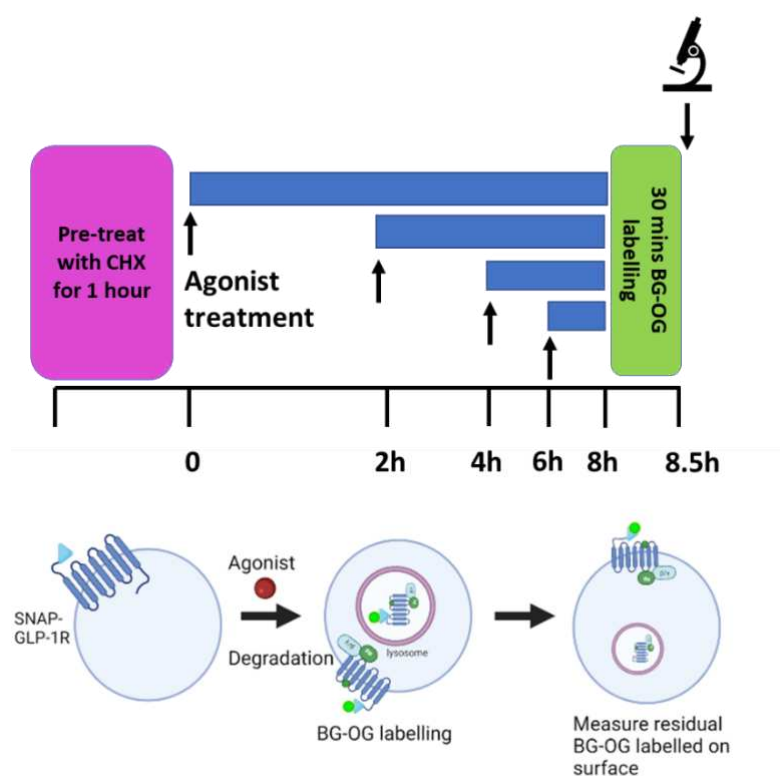


Figure 2.3 Scheme representing degradation assays performed in INS-1 832/3 SNAP-hGLP-1R^{WT} or hGLP-1R^{A316T} cells

2.2.5 NanoBiT complementation assays

NanoBiT mini-G_s and β -arrestin 2 GLP-1R recruitment assays

To measure mini-G_s / β -arrestin 2 recruitment to the WT GLP-1R *versus* the A316T GLP-1R, INS-1 832/3 *Glp1r*^{-/-} cells were seeded in 6-cm dishes and co-transfected for 24 hours using Lipofectamine 2000 with the following quantities of DNA: 1.7 μ g each of *hGlp1r*^{WT}-SmBiT or *hGlp1r*^{A316T}-SmBiT and LgBiT-mini-G_s/mini-G_i/ β -arrestin 2 plasmid, for 24 hours. To perform the assay, cells were detached and resuspended in NanoGlo Live Cell Reagent (Promega), which contains furimazine, in HBSS buffer in a 1:1:0.05 ratio and seeded into white 96-well half area plates. Baseline luminescence was immediately recorded over 8 minutes at 37°C in a Flexstation 3 plate reader (Molecular Device, UK), and consecutively after agonist addition at serial doses of up to 100 nM (mini-G_s) or 1 μ M (β -arrestin 2) for 30 min. Readings were taken every 30 seconds and responses were normalised to average baseline and then to average vehicle-induced signal to establish the agonist-induced effect. Areas under the curve (AUCs) were calculated for each agonist concentration and fitted to log(agonist) *versus* response four-parameter curves using GraphPad Prism 10.1.2.

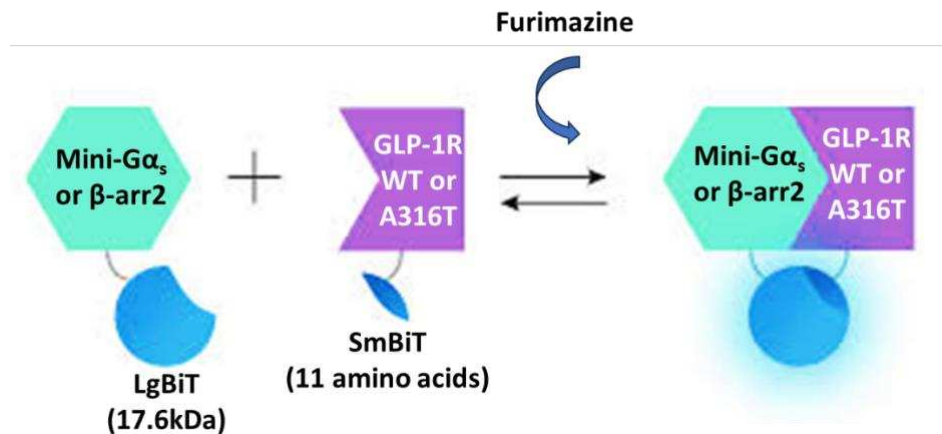


Figure 2.4 Schematic representation of the genetically encoded nanosensor principle. A large NanoLuc Binary Technology (NanoBiT) subunit (LgBiT) and small NanoBiT subunit (SmBiT) are genetically fused to mini- $G\alpha_s$ protein or β -arrestin 2 and hGLP-1R^{WT} or hGLP-1R^{A316T}, respectively. Recruitment of mini- $G\alpha_s$ or β -arrestin 2 (β -arr2) by the GLP-1R WT or A316T causes reconstitution of the NanoBiT luciferase, which in turn will emit light after the addition of the furimazine substrate.

Nb37 bystander NanoBiT assays

The same cells mentioned above were seeded in 6-well plates and different wells were co-transfected for 24 hours using Lipofectamine 2000 with the following DNA quantities: 200 ng pSNAP/FLAG-*hGlp1r*^{WT} or pSNAP/FLAG-*hGlp1r*^{A316T}, 500 ng $G\alpha_s$ (human, short isoform), 500 ng $G\beta_1$ (human), 500 ng $G\gamma_2$ (human), and 100 ng RIC8B (human, isoform 2), and either 100 ng CAAX-LgBiT (C-terminal KRAS CAAX motif C-terminally fused with LgBiT or 500 ng Endofin-LgBiT (Endofin FYVE domain C-terminally fused with LgBiT), plus 100 ng (plasma membrane assay) or 500 ng (endosomal assay) of SmBiT-Nb37 (a construct consisting of Nb37 cDNA synthesised by GenScript C-terminally fused to SmBiT with a 15-amino-acid flexible linker) and topped up with pcDNA3.1 for the LgBiT-CAAX transfection. 24 hours after transfection, the assay was performed as before in NanoGLO Live Cell Reagent (Promega) with furimazine (1:20 dilution) and read in a Flexstation 3 plate reader, with stimulation by serial doses of up to 1 μ M GLP-1. LogEC50 (potency) and Emax (efficacy)

were recorded for each experiment, as well as a ratio of the endosomal *versus* plasma membrane luminescence signal for wild-type and variant receptors.

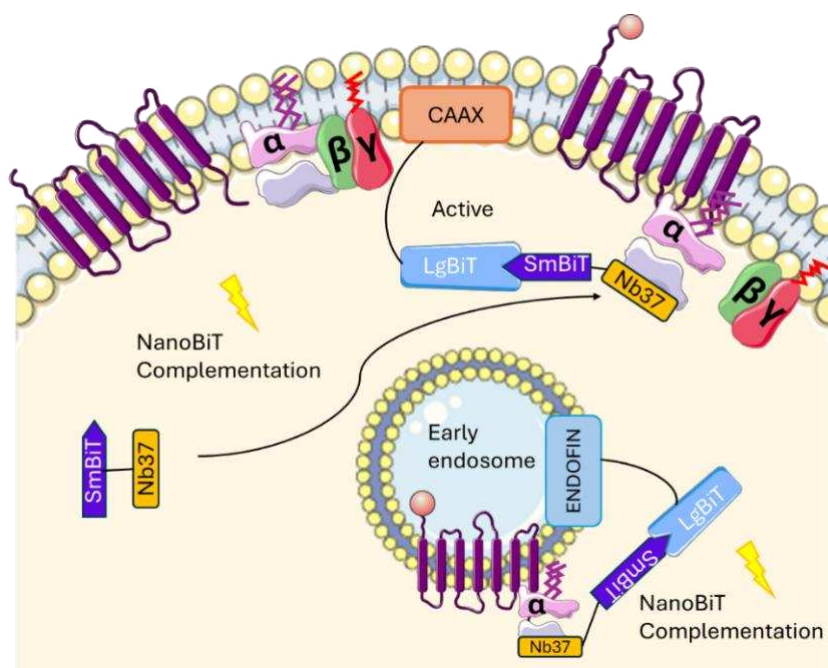


Figure 2.5 Schematic representation of NanoBiT subcellular G protein activation assay. The hGLP-1R^{WT} or hGLP-1R^{A316T} is quiescent at the cell membrane. When an agonist binds to its receptor, Gα_s is recruited and activated binding to Nb37. This Nb37-SmBiT complements CAAX-LgBiT upon plasma membrane activation. Following endocytosis of the receptor, Nb37-SmBiT complements with Endofin-LgBiT indicating endosomal signalling.

2.2.6 Time-Resolved Fluorescence Resonance Energy Transfer (TR-FRET) assays

Measurement of receptor clustering by TR-FRET

The time-resolved fluorescence resonance energy transfer (TR-FRET) assay was performed as previously described. INS-1 832/3 SNAP-hGLP-1R^{WT} and SNAP-hGLP-1R^{A316T} cells were labelled in suspension with 40 nM SNAP-Lumi4-Tb and 1 mM SNAP-Surface 649 (New England Biolabs, Hitchin, UK) for 1 hour at room temperature in complete medium. After washing, cells were resuspended in HBSS and TR-FRET was recorded before and after

stimulation by serial doses of up to 100nM GLP-1 at 37 °C in a Spectramax i3x plate reader in homogeneous time-resolved fluorescence (HTRF) mode. TR-FRET was quantified as the ratio of fluorescent signal at 665nm to that at 616nm, after subtraction of background signal at each wavelength.

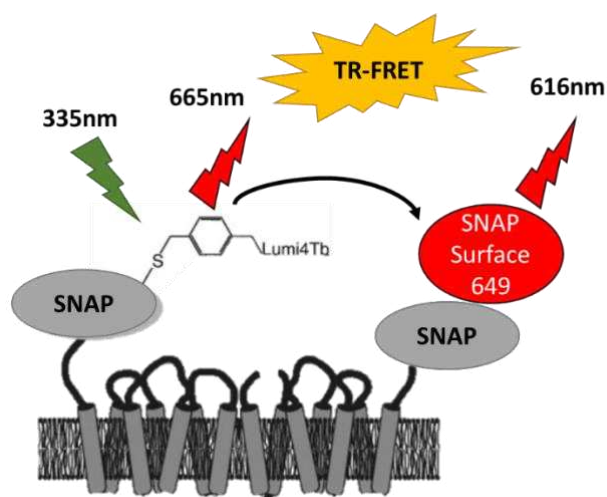


Figure 2.6 Schematic showing the principle of the TR-FRET clustering assay. Showing how close proximity between SNAP-hGLP-1R^{WT} and SNAP-hGLP-1R^{A316T} receptors dual labelled with Lumi-4Tb and SNAP-Surface 649 probe leads to an increase in energy transfer from one probe to another, detected as an increase in TR-FRET.

NR12A conformational sensor assay

INS-1 832/3 SNAP-hGLP-1R^{WT} and SNAP-hGLP-1R^{A316T} cells were labelled with SNAP-Lumi4-Tb (40 nM, 60 min at 37 °C, in complete medium). After washing to remove unbound probe, cells were resuspended in HBSS ± 100 nM NR12A (a gift from Prof Andrey Klymchenko, University of Strasbourg) for 5 minutes before washing again. Labelled cells were transferred to 96-well half area white plates and baseline TR-FRET signal from Lumi-4Tb (donor) and NR12A (acceptor) were recorded for 5 min at 37 °C using a Flexstation 3 plate reader with the following settings: $\lambda_{\text{ex}} = 335 \text{ nm}$, $\lambda_{\text{em}} = 490 \text{ and } 590 \text{ nm}$, delay 50 μs ,

integration time 300 μ s. GLP-1 was then added at serial doses, and signal was serially monitored for 30 min. The TR-FRET ratio, i.e. the ratio of fluorescence intensities at 590 and 490 nm, was considered indicative of the proximity of the GLP-1R extracellular domain (ECD) to the plasma membrane. Baseline-normalised AUCs were used for concentration–response analysis.

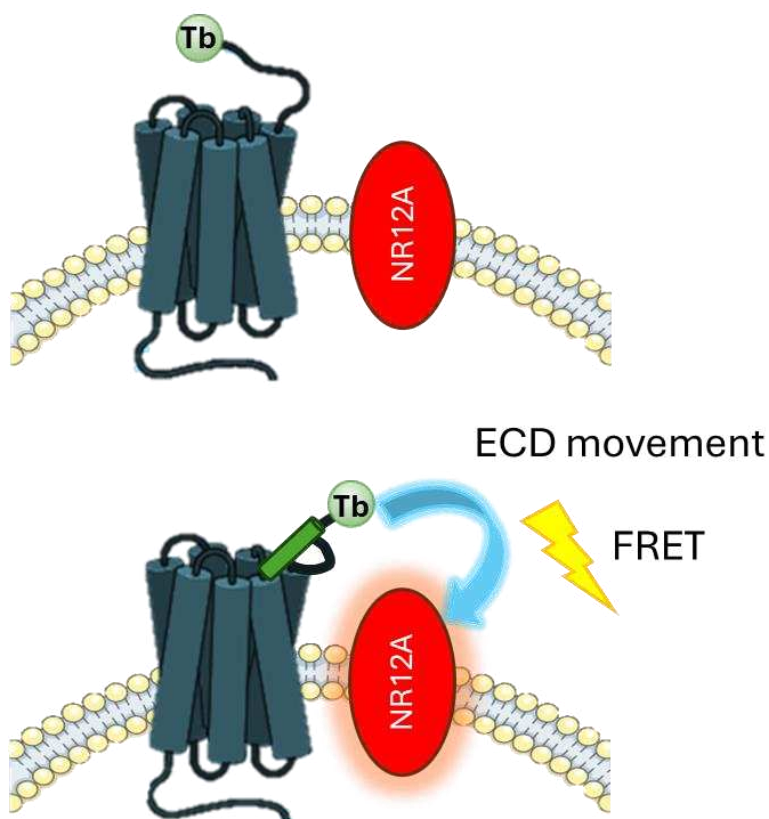


Figure 2.7 Schematic showing the principle of the TR-FRET NR12A conformational assay. Ligand-induced Lumi4-Tb labelled SNAP-hGLP-1R^{WT} or SNAP-hGLP-1R^{A316T} ECD movement leads to an increase in energy transfer to the plasma membrane probe NR12A, detected as an increase in TR-FRET.

2.2.7 Raster Image Correlates Spectroscopy (RICS) image capture analysis

INS-1 832/3 SNAP-hGLP-1R^{WT} or hGLP-1R^{A316T} cells were seeded onto 35-cm glass bottom MatTek dishes and labelled with SNAP-Surface Alexa Fluor 647 for 10 minutes at 37°C in

full media, washed and imaged in RMPI-1640 media without phenol red using a 100x oil objective on a Leica Stellaris 8 STED FALCON microscope from the Imperial College FILM Facility, using confocal settings. Cells were imaged and analysed as previously described (539), at the basal plasma membrane, both under vehicle conditions and following stimulation with 100 nM exendin-4 with a format size of 256 x 256 pixels and 80 nm pixel size for 200 consecutive frames. RICS analysis was carried out using the SimFCS 4 Software (Global Software, G-SOFT Inc) to determine the diffusion coefficient of hGLP-1R^{WT} versus hGLP-1R^{A316T} in vehicle and exendin-4-stimulated conditions. The RICS experiments were performed by Dr. Affiong Ika Oqua and Mrs. Yusman Manchanda.

2.2.8 Real-time measurement of cAMP Generation using cADDIs and FRET biosensors

cAMP FRET imaging using global and endosomal- targeted ^TEPAC^{VV}

The globally targeted Epac and mTurquoise-based ^TEPAC^{VV} FRET biosensor construct was a gift from Prof. Kees Jalink, the Netherlands Cancer Institute, Netherlands. The endosome-targeted ^TEPAC^{VV} biosensor (Endo-^TEPAC^{VV}) was generated by PCR cloning of the 2xFYVE domain (endosomal targeting sequence) of the bPAC-Endo construct (a kind gift from Prof Mark Von Zastrow, UC San Francisco) to the N-terminus end of the ^TEPAC^{VV} using HindIII and NotI restriction sites. INS-1 832/3 SNAP-hGLP-1R^{WT} and SNAP-hGLP-1R^{A316T} cells were plated in 48 wells and transfected with 0.5 µg of plasmid DNA. After 24 hours, cells were trypsinised and seeded onto glass-bottom dishes. Forty-eight hours after transfection, cells were imaged in RPMI 1640 without phenol red (32404014, ThermoFisher Scientific) with an NHLI FILM Zeiss LSM-780 inverted confocal laser-scanning microscope using a 20×

objective to capture a time-lapse recording of CFP/YFP FRET with image acquisition every 6s and treatments added manually by pipetting. Cells were imaged for 1 min to record a baseline, and then 100nM exendin-4 was added and imaged for 5 min before addition of a mixture of 100 μ M isobutyl methylxanthine (IBMX) and 10 μ M forskolin (FSK) for the final 2 min of the acquisition. Raw intensity traces for YFP and CFP fluorescence were extracted from individual cell ROIs using ImageJ v1.541c, and CFP/YFP ratios were calculated for each ROI and time point. Responses were plotted relative to the average fluorescence intensity during the 6mM glucose baseline period, before agonist addition.

cAMP imaging with cADDis

INS-1 832/3 SNAP-hGLP-1R^{WT} and SNAP-hGLP-1R^{A316T} cells were seeded onto 35-mm glass-bottom dishes (MatTek) and incubated overnight with the Green Up cADDis biosensor in a BacMam vector (Montana Molecular) according to the manufacturer's instructions. Cells were washed with PBS and imaged in 11 mM glucose Krebs-Ringer Bicarbonate HEPES (KRBH) buffer (140 mM NaCl, 3.6 mM KCl, 1.5 mM CaCl₂, 0.5 mM MgSO₄, 0.5 mM NaH₂PO₄, 2 mM NaHCO₃, 10 mM HEPES, saturated with 95% O₂/5% CO₂; pH 7.4) containing 0.1% using a Nikon Eclipse Ti microscope with an ORCA-Flash 4.0 camera (Hamamatsu) and Metamorph software (Molecular Devices) with a 20x objective and 488 nm laser with images taken every 6 seconds on a 37°C stage. A baseline reading was taken for 1 min followed by stimulation with 100 nM GLP-1R agonist for 5 minutes, and a mixture of 100 μ M IBMX and 10 μ M FSK for the last 2 minutes of the acquisition. Raw fluorescent intensities were extracted in Fiji (1.541c) to plot an average intensity trace and AUCs were calculated GraphPad Prism 10.

2.2.9 Real-time imaging of cytosolic calcium

INS-1 832/3 SNAP-hGLP-1R^{WT} and SNAP-hGLP-1R^{A316T} cells were plated into 35-cm glass bottom dishes as for section 2.2.8. Cells were then incubated with Ca²⁺ responsive dye Cal-520 AM (4.5 μ M; Stratech) diluted in KRBH buffer (140 mM NaCl, 4.8 mM KCl, 1.25 mM NaH₂PO₄, 24 mM NaHCO₃, 5 mM HEPES, 2.5 mM CaCl₂ and 1.2 mM MgCl₂; Bubbled with 95 % O₂/5 % CO₂ for ~20 minutes and adjusted to pH 7.4) containing 0.1% (w/v) BSA and 6 mM glucose (Sigma Aldrich) for 1 hour. Cells in the MatTek glass bottom dishes were then imaged every 6 seconds at 488 nm using a Nikon Eclipse Ti microscope with an ORCA-Flash 4.0 camera (Hamamatsu) and Metamorph software (Molecular Devices) while maintained at 37 °C on a heated stage. Raw fluorescence intensity traces from cell-occupied areas or islet ROIs were extracted using Fiji. Responses were plotted relative to the average fluorescence intensity during the 6 mM glucose baseline period, before agonist addition.

2.2.10 Isolation of detergent-resistant and detergent-soluble membranes

To purify lipid membrane rafts, detergent-resistant membrane fractions (DRFs) were isolated by ultracentrifugation based on their insolubility in non-ionic detergents at 4°C and their low density because of their high lipid-to-protein ratio. Initially, INS-1 832/3 SNAP-hGLP-1R^{WT} and SNAP-hGLP-1R^{A316T} cells were seeded onto 6-cm dishes. After treatment with complete INS-1 medium with or without 100 nM exendin-4 or GLP-1 for 2 minutes, cells are washed with ice-cold PBS and osmotically lysed in cold low salt buffer 20 mM Tris-HCl (pH7.0) supplemented with cOmplete EDTA-free protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail 2 (Sigma-Aldrich) for 10-15 minutes on a rocking platform at 4°C. The cell

suspension was then collected in clean Eppendorf tubes and homogenized by passing through a 21-gauge needle, followed by ultracentrifugation at 63,000 g for 1 hour at 4 °C with a Sorvall Discovery M120 ultracentrifuge. The supernatant was then discarded, and the pellet was resuspended in 200 µl ice-cold PBS supplemented with cOmplete EDTA-free protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail 2 and 1% Triton X-100, followed by incubation on a rotor for 30 minutes at 4 °C. The detergent-treated membrane fraction is then transferred back to ultracentrifuge tubes and centrifuged again at 63,000 g for 1 hour at 4 °C. The supernatant, containing the ‘disordered’ detergent-soluble membrane fraction (DSF), was removed and retained for analysis, while the remaining ‘ordered’ detergent-resistant insoluble membrane fraction (DRF) pellet was resuspended in 1% SDS containing cOmplete EDTA-free protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail 2 (Sigma Aldrich). All samples were then sonicated with the Ultrasonic cell disruptor XL-2000 probe sonicator (Misonix, Inc) and centrifuged at 14,000 g for 5 minutes at 4 °C before western blot analysis.

2.2.11 Anti-Flag M2 affinity purification and LC-MS/MS

INS-1 832/3 SNAP-hGLP-1R^{A316T} cells (which also include an N-terminal FLAG tag) were stimulated with vehicle or 100 nM GLP-1RA (GLP-1 and exendin-4) for 5 minutes as previously described (540). Following stimulation, a 6-cm dish of cells from each condition was washed with PBS and osmotically lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA) with 1% protease inhibitor (Roche) and 1% phosphatase inhibitor cocktail (Sigma-Aldrich) at 4°C. The cell lysates were centrifuged at 12,000 g and 4°C for 10 minutes, prior to the addition of 40 µl of anti-FLAG M2 beads (A2220,

Sigma-Aldrich) for overnight rotating incubation at 4°C to increase binding efficiency. Samples were centrifuged at 5,000 g for 30 seconds, and bead pellets washed three times in 500 µl wash buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4), before storage at -80°C until further analysis.

Beads were resuspended in 150 µl 1 M urea in 20 mM HEPES pH 8.0, 1 mM dithiothreitol (DTT) followed by addition of 1.5 µg of Trypsin gold (Promega) with overnight digestion at 37°C. Supernatants were subsequently acidified with 0.5% trifluoroacetic acid (TFA), followed by desalting using reversed-phase spin tips (Glygen Corp) and dried using vacuum centrifugation. Dried peptides were resuspended in 0.1% TFA and Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) performed as previously described (541) using an Ultimate 3000 nano-HPLC coupled to a Q-Exactive mass spectrometer (Thermo Scientific) via an EASY-Spray source. Peptides were loaded onto a trap column (Acclaim PepMap 100 C18, 100 µm × 2 cm) at 8 µl/minute in 2% acetonitrile, 0.1% TFA. Peptides were then eluted on-line to an analytical column (Acclaim Pepmap RSLC C18, 75 µm × 75 cm). A 90-minute stepped gradient separation was used with 4-25% acetonitrile 80%, formic acid 0.1% for 60 minutes, followed by 25-45% for 30 minutes. Eluted peptides were analysed by Q-Exactive operating in positive polarity and data-dependent acquisition mode. Ions for fragmentation were determined from an initial MS1 survey scan at 70,000 resolution (at m/z 200), followed by higher-energy collisional dissociation of the top 12 most abundant ions at a resolution of 17,500. MS1 and MS2 scan AGC targets were set to 3e6 and 5e4 for a maximum injection time of 50 mseconds and 75 mseconds, respectively. A survey scan m/z range of 350 – 1800 m/z was used, with a normalised collision energy set to 27%, minimum AGC of 1e3, charge state exclusion enabled for unassigned and +1 ions and a dynamic exclusion of 45 seconds.

Data was processed using the MaxQuant software platform (v1.6.10.43) (542), with database searches carried out by the in-built Andromeda search engine against the Swissprot Rattus Norvegicus database (Downloaded – 21st May 2022, entries: 8,132) concatenated with the human GLP-1R protein sequence. A reverse decoy database approach was used at a 1% FDR for peptide spectrum matches and protein identifications. Search parameters included: maximum missed cleavages set to 2, variable modifications of methionine oxidation, protein N-terminal acetylation, asparagine deamidation and cyclisation of glutamine to pyroglutamate. Label-free quantification was enabled with an LFQ minimum ratio count of 1. Proteins were filtered for those containing at least one unique peptide and identified in all 3 biological replicates. For normalisation, proteins were ranked by fold change increase in abundance compared with control (vehicle-treated) immunoprecipitates. The resulting data was pre-processed by elimination of proteins that are non-specifically bound and residual background proteins, such as common contaminants and reverse database proteins. To further facilitate the selection of GLP-1R interactors, the following criteria were established; i) identified at least twice from four runs; ii) not nuclear proteins; and (iii) previously identified in pancreatic β -cells.

Untargeted label-free quantitation (LFQ) intensity values, normalised to hGLP-1R levels in each immunoprecipitated sample, were analysed by LFQ Analyst to identify differences in levels of individual protein-GLP-1R interactions between vehicle-and GLP-1RA-treated conditions (543). Paired t-tests adjusted for multiple comparisons using Benjamin-Hochberg false discovery rate correction and Perseus type imputations were used for statistical analysis.

2.2.12 Co-Immunoprecipitation for ubiquitination assay

INS-1 832/3 SNAP-hGLP-1R^{WT} and A^{316T} cells were seeded and processed as above with the anti-FLAG M2 beads overnight. However, after washing three times with wash buffer, proteins were eluted with 2x urea buffer (200 mM Tris-HCl, pH 6.8; 5% w/v SDS, 8 M urea, 100 mM DTT, 0.02% w/v bromophenol blue) at 37°C for 10 minutes before Western blot analysis.

2.2.13 Protein extraction and Western Blotting

To quantify SNAP-GLP-1R protein levels in INS-1 832/3 SNAP-hGLP-1R^{WT} and A^{316T} cells, these were stimulated with vehicle or exendin-4 for 5 minutes. Cells were then washed once in ice-cold PBS and osmotically lysed with lysed 1x TNE lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% NP40, protease and phosphatase inhibitors) for 10 minutes at 4 °C. Samples were then sonicated in a water bath sonicator three times for 10 seconds, prior to centrifugation at 13,000 rpm for 5 minutes. Supernatants were subsequently collected, and proteins were eluted with 2x urea buffer (200 mM Tris HCl, 5% w/v SDS, 8 M urea, 100 mM DTT, 0.02% w/v bromophenol blue, pH 6.8).

To resolve proteins in the samples by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), samples were first loaded onto 10% SDS-polyacrylamide gels then transferred to PVDF membranes (Immobilon-P, 0.45-µm pore size, IPVH00010, Merck) using a Bio-Rad wet transfer system, before blocking with 5% skimmed milk in TBS buffer supplemented with 0.1% Tween 20 (TBS-T) for 1 hour. Membranes were then incubated with the appropriate primary antibody in 5% milk/TBS-T buffer overnight at 4°C, washed in TBS-T and incubated with the appropriate HRP-conjugated secondary antibody in 5% milk/TBS-T for 1 hour at

room temperature. Membranes were then washed again three times with TBS-T, prior to being developed with Clarity Western ECL substrate (BioRad, California, USA) and imaged on Biorad ChemiDoc or in a Xograph Compact X5 processor with Fuji medical X-ray films. Specific band densities were quantified in Fiji v1.54i. Details for the primary antibodies and respective secondary antibodies are provided in Table 2.2.

Table 2.2 List of antibodies used during this PhD project

Name	Species	Type of Antibody	Source	Dilution/Concentration
Anti-SNAP tag	Rabbit	Primary	New England Biolabs	1:500
Anti-flotillin 1	Mouse	Primary	Sc-74566, Santa Cruz Biotechnology	1:200
Anti- α -Tubulin	Mouse	Primary	T5168, Sigma	1:1000
Anti-insulin	Guineapig	Primary	IR002, Dako, Agilent, USA	1:50
Anti-glucagon	Mouse	Primary	G2654, Sigma-Aldrich, UK	1:500
Anti-ubiquitin	Mouse	Primary	Santa Cruz Biotechnology	1:1000
IgG-HRP	Mouse	Secondary	Abcam	1:5000
IgG-HRP	Rabbit	Secondary	Abcam	1:2000
Alexa Fluor 488	Guineapig	Secondary	ThermoFisher Scientific	1:500
Alex Fluor 647	Mouse	Secondary	ThermoFisher Scientific	1:500

2.2.14 Titration and transduction with SNAP-*hGlp1r*^{WT} or SNAP-*hGlp1r*^{A316T} adenoviruses

The pSNAP/FLAG-*hGlp1r*^{WT} and *hGlp1r*^{A316T} plasmid vectors were used to generate high-titre ultrapure recombinant adenovirus particles for experiments in islets and human β -cells by VectorBuilder Inc. (Chicago, IL, USA). The functional titre was measured by using serially diluted adenovirus transduction into HEK293 cells followed by an immunocytochemistry-based approach to count the number of cells being successfully transduced via the detection of adenovirus-specific hexon protein, and each immunostained cell is considered as one infectious unit (IFU). Further validation is carried out by measuring the optical density of the viral particles which is tightly correlated to purified adenovirus titre. The viral IFU/ml for pAV-CMV-SNAP-*hGlp1r*^{WT} (Vector ID: VB220107-1221xbv) was 2.99×10^{10} IFU/ml and 2.01×10^{10} IFU/ml for pAV-CMV-SNAP-*hGlp1r*^{A316T} (Vector ID: VB220107-1223tqy). Using a multiplicity of infection (MOI) of 1, islets and EndoC- β H3 cells were infected with SNAP-*hGlp1r*^{WT} or ^{A316T} for 24-48 hours prior to experiments.

2.2.15 Glucose-stimulated insulin secretion in EndoC- β H3

EndoC- β H3 cells were transduced with pAV-CMV-SNAP-*hGlp1r*^{WT} or ^{A316T} adenoviruses as above after three weeks of 1 μ M tamoxifen treatment to excise the immortalizing transgenes transforming cells into functional non-proliferative human β -cells (544). Cells were then seeded in triplicate in 24-well fibronectin-coated plates and pre-incubated overnight in 2.8 mM glucose medium. The following day, cells were incubated in Krebs-Ringer Bicarbonate (KRB) buffer (460 mM NaCl, 20mM KCL, 4 mM CaCl₂, 4mM MgCl₂, 96 mM NaHCO₃ and 0.2% BSA; saturated with 95% O₂/5% CO₂; pH 7.4) supplemented with 0.5 mM glucose and then

incubated for 1 hour at 37°C in KRB buffer supplemented with 15 mM glucose $\pm$ 100 nM exendin-4. Supernatants were then harvested to determine stimulated insulin secretion. Cells were lysed in KRB buffer + 1% Triton X-100 and lysates collected to determine cellular insulin content. Total and secreted insulin levels were measured using an HTRF-based insulin detection assay (Insulin Ultra-Sensitive Assay Kit, Cisbio Bioassays, 62IN2PEG) in a PHERAstar reader (BMG Labtech), following the manufacturer's protocol. The percentage of insulin secreted was calculated and agonist-stimulated results were expressed alone as well as a fold change relative to 15 mM glucose alone.

2.2.16 Insulin Secretion homogenous time-resolved fluorescence assays

Internal and secreted insulin were measured from samples by HTRF. The assay relies on two insulin binding (anti-insulin) antibodies, one antibody labelled with Europium cryptate (donor), and anti-Insulin antibody labelled with XL665 (a near-infrared acceptor). When both antibodies are bound to insulin in samples, they are in close enough proximity for FRET to occur in the presence of a light source exciting the donor. In the absence of insulin, no FRET occurs and only the donor will emit a signal at 620 nm, but in the presence of insulin FRET occurs towards the acceptor allowing the detection of additional emission at 665nm. Signal intensity is directly proportional to the number of insulin-antibody complexes formed and therefore to insulin secretion.

2.2.17 Generation of EndoC-βH3 GLP-1R knockout cell line

For knock-out of GLP-1R in EndoC-βH3 cells, a lentiviral CRISPR approach was used (517). Two guide RNA (gRNA) sequences were cloned using a pScaffold-H1 (118152, Addgene) into a lenti-CRISPRv2-Blast backbone (83480, Addgene) which allows positive selection of EndoC-βH3 cells after transfection with blasticidin. Cas-Designer (<http://www.rgenome.net/cas-designer/>) to design 17nt-long (truncated) guide RNAs (gRNAs) 38 flanking the target DNA sequences. The gRNA sequences were: 5'-GCTGCTCGGGATGGTGGGCA-3', and 5'-GTTGCAGAACAAGTCTGTGG-3' (reverse complement). The dual guide RNA cloning strategy used has been described previously (545, 546). Briefly, oligonucleotides corresponding to deletion-yielding pairs of gRNAs were assembled in a reaction mix containing 1X Q5 reaction buffer, 200 μM dNTPs, 0.5 μM primer, 0.02U/μl Q5 high-fidelity DNA polymerase and 0.25 ng/μl of pScaffold-H1 vector (plasmid #118152, Addgene), with Ta=58°C, 15 seconds extension, for 30 amplification cycles. PCR products were digested and ligated to lentiCRISPRv2-Blast in a one-step digestion ligation reaction with 1X tango buffer, 1μl FastDigest BsmBI/Esp3I (ThermoFisher Scientific), 1 μl 0.1 M DTT, 1 μl 10 mM ATP, 0.5 μl T7 ligase, 100 ng of vector and 1 μl of PCR product (diluted 1:20). Digestion-ligation reactions underwent 6 cycles of 5 minutes at 37°C and 5 minutes at 23°C, and a final 5 minute incubation at 37°C, after which 1 μl of the final ligation was transformed into NEB Stable competent E. coli cells (New England Biolabs, UK) and plated on ampicillin resistant plates. Successful gRNA insertion was confirmed with Sanger Sequencing.

Lentiviral production and transduction into EndoC- β H3

To produce lentiviral particles, HEK293T cells were co-transfected with gRNA lentiviral vector as well as the packaging (psPAX2) and envelope (pMD2.G) vectors using a calcium phosphate protocol (547). Viral supernatants were harvested 48 and 72 hours after transfection, filtered using a 0.45- μ m Millex-HV filter, and concentrated by adding PEG 8000 (Sigma) and NaCl until final concentrations of 10% and 0.15 M, respectively. The mixture was then incubated for 16-20 hours on a shaker at 4 °C, followed by centrifugation for 30 minutes at 10,000 g at 4 °C. Virus pellet was resuspended in 100 μ l with PBS and stored at -80°C until use. 48 hours before transduction, EndoC- β H3 cells were seeded at 1,000,000 cells per 6-well plate, after which 50 μ l of virus (a high MOI of 8) was used to infect one well of plated cells to generate the EndoC- β H3 KO cell line. After 72 hours, cells were selected for vector integration using blasticidin (20 μ g/ml) for 10 days. Medium was changed every ~3 days to remove uninfected cells and to maintain fresh blasticidin during culture. After selection, cells were further sorted by labelling with the fluorescent exendin-9-TMR for 10 minutes and FACS sorted for the non-labelled cells. After sorting, cells were cultured in

regular medium and passaged weekly. CRISPR-Cas9-mediated *Glp1r* deletion efficiency was assessed by functional HTRF cAMP assay.

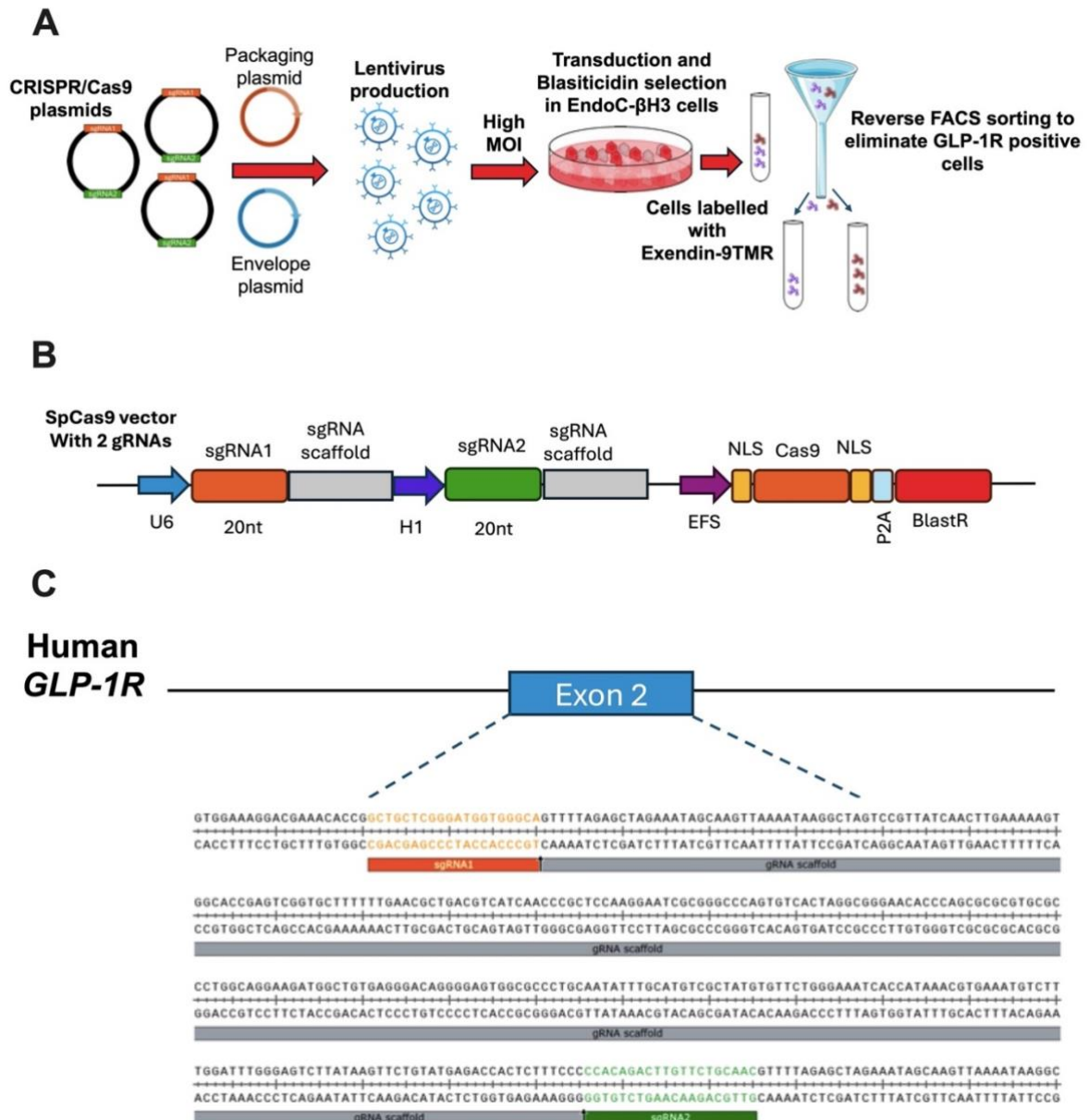


Figure 2.8 Generation of a GLP-1R knockout EndoC-βH3 human β cell line via a lentiviral CRISPR/Cas9 pipeline. A) Strategy to genome edit EndoC-βH3 human β-cell line using a lentiviral dual sgRNA approach resulting in a population with cells lacking *Glp1r* gene expression. B) CRISPR plasmid, lenti-CRISPR v2-Blast containing Cas9 and two sgRNAs. C) Gene targeting strategy using two sgRNA to target exon 2 of the human *Glp1r*.

2.2.18 Homogenous time-resolved fluorescence cAMP assays for functional characterisation of the EndoC- β H3 GLP-1R knockout cell line

EndoC- β H3 and EndoC- β H3 GLP-1R KO cells were pre-treated with tamoxifen and seeded as mentioned in section 2.2.15. Then on the day of the experiment cells were incubated in 0.5 mM EndoC- β H3 glucose medium $\pm$ 100 nM exendin-4 and the phosphodiesterase inhibitor IBMX, as indicated. Subsequently, cells were lysed in KRB buffer + 1% Triton X-100 and lysates were collected to determine cellular cAMP content. cAMP production was then measured using a cAMP Dynamic 2 HTRF assay kit (Cisbio Assays) in a PHERAstar reader (BMG Labtech), following the manufacturer's instructions.

2.3 *Ex vivo* experiments

2.3.1 Isolation and maintenance of human islets

Our laboratory regularly receives human islets isolated from deceased heart-beating donors from the European Diabetes Centre (CEED) facilities in Strasbourg, after obtaining written informed consent from donor or family members (Table 2.3). The study was conducted in accordance with the Human Tissue Act (HTA) 2004, and the protocol was approved by the Imperial College London ethics committee. Human islets were cultured in RPMI-1640 medium with L-glutamine (Sigma-Aldrich), 10% (v/v) FBS, 1% (v/v) P/S, 0.25 μ g/mL Amphotericin B (Sigma-Aldrich), and 5.5 mM D-glucose at 37°C in 5% CO₂. The characteristics of the human donors are shown in Table 2.3.

Table 2.3 Donor Characteristics of human islets used during this PhD project.

Arrival date	Donor Age	Sex	BMI (kg/m²)
24.11.21	73	Female	32
08.12.21	46	Female	31.9
10.12.21	67	Male	29.7
08.02.22	64	Male	26.7

2.3.2 Isolation and maintenance of mouse islets

The *hGlp1r*^{A316T/A316T} mice, *hGlp1r*^{+/+} littermate controls and *hGlp1r*^{-/-} mice were humanely euthanised by cervical dislocation of the neck followed by decapitation prior to pancreas inflation via injection of RPMI-1640 medium (Sigma-Aldrich) containing 1 mg/ml collagenase NB8 from *Clostridium histolyticum* (S1745602, Nordmark Biochemicals) into the common bile duct. The inflated pancreas was then dissected and incubated in a water bath to digest at 37°C for 10 minutes. The digestion of the pancreas was quenched with cold mouse islet culture medium: RPMI 1640 medium containing 10% (v/v) foetal bovine serum (FBS) (F7524, Sigma-Aldrich, UK) and 1% (v/v) penicillin/streptomycin (P/S) solution (15070-063, Invitrogen). Subsequently, the digested pancreata was washed three times with RPMI-1640 (Sigma-Aldrich), before islets were purified using a Histopaque gradient (Histopaque-1119 and -1083, Sigma-Aldrich, UK). Islets were then allowed to recover overnight at 37°C in 5% CO₂ in mouse islet culture medium, as mentioned above, prior to any experiments.

2.3.3 Glucose-stimulated insulin secretion in mouse and human islets

Mouse or human islets (8-10 islet) were treated per well of a 24-well non adherent plate, with three replicates per condition for GSIS assays. For acute studies (short-term), islets were preincubated for 1 hour in KRBH buffer (140 mM NaCl, 3.6 mM KCl, 1.5 mM CaCl₂, 0.5 mM MgSO₄, 0.5 mM NaH₂PO₄, 2 mM NaHCO₃, 10 mM HEPES, saturated with 95% O₂/5% CO₂; pH 7.4) containing 0.1% (w/v) bovine serum albumin (BSA) (10775835001, Roche) and 3 mM glucose before incubation with 11 mM glucose $\pm$ 100 nM agonists in KRBH in a shaking 37°C water bath (80 rpm) for 1 hour to induce insulin secretion. For overnight (long-term) studies, pre-incubation was carried out in RPMI 1640 medium containing FBS, penicillin/streptomycin, and 3 mM glucose, followed by treatment with medium containing 11 mM glucose $\pm$ agonists for 16 hours. Supernatant containing secreted insulin was collected, centrifuged at 1000 rpm for 1 minute, and transferred to fresh tubes for analysis. Islets were lysed in acidic ethanol (75% ethanol and 1.5 mM HCl) for total insulin content. Lysates were subsequently sonicated three times for 10 seconds each and centrifuged at 10,000 g for 10 min, and supernatants were collected. For human islets and *hGlp1r*^{-/-} mouse islets the same experiment was performed with the modification that islets were transduced with pAV-CMV-SNAP-*hGlp1r*^{WT} or *hGlp1r*^{A316T} adenoviruses as described above, 24 hours prior to start of experiments. Total and secreted insulin samples were stored at -20°C until quantification using an Insulin Ultra-Sensitive HTRF kit (62IN2PEG, Cisbio, Codolet, France) in a PHERAstar reader (BMG Labtech), according to the manufacturer's instructions as mentioned in section 2.2.16.

2.3.4 Islet calcium imaging

Islet calcium imaging was performed as previously described(362, 548). Briefly, *hGlp1r*^{-/-} mouse islets transduced with pAV-CMV-SNAP-*hGlp1r*^{WT} or *hGlp1r*^{A316T} adenoviruses were pre-incubated for 1 hour in KRBH buffer, containing 0.1% (w/v) BSA, 6mM glucose (KRBH G6) and the Ca²⁺-responsive dye Cal-520 AM (4.5 μM, AAT Bioquest). Islets were then encased in Matrigel (356231, Corning) to ensure that they remained stable, and were then imaged in MatTek glass bottom dishes (P35G-1.5-10-C, MatTek) every 6 seconds at 488nm using a Nikon Eclipse Ti microscope with a 20x objective, an ORCA-Flash camera (Hamamatsu) and Metamorph software (Molecular Devices) while maintaining a constant temperature of 37°C on a heated stage. Treatments were added manually to the islet dishes by pipetting at the indicated time points. Raw fluorescence intensity traces from whole-islet ROIs were extracted and analysed using Fiji (v1.54i). Responses were plotted relative to the average fluorescence intensity during the 6mM glucose baseline period, prior to agonist administration.

2.3.5 Islet cAMP imaging with cADDIs

To evaluate cAMP levels, islets from *hGlp1r*^{A316T/A316T} or *hGlp1r*^{+/+} control mice were transduced with cADDIs (Green Gs cADDIs cAMP Kit, Montana Molecular), a baculovirus that encodes a fluorescent cAMP biosensor, for 24 hours prior to the experiment, according to the manufacturer's guidelines. The transduced islets were then encapsulated in Matrigel and placed on glass-bottom dishes, for imaging at 488 nm in a Nikon Eclipse Ti Spinning disk confocal microscope at a 20x objective with an ORCA-Flash 4.0 camera (Hamamatsu) for time-lapse recordings capturing images every six seconds. The islets were imaged in KRBH

buffer containing 0.1% (w/v) BSA and 6 mM glucose (KRBH G6) for an initial two minute to establish a baseline, followed by the addition of 100 nM GLP-1RA (GLP-1, exendin-4, exendin-F1, exendin-D3, semaglutide) via manual pipetting. The islets were then imaged for an additional five minutes before the addition of a mixture of 100 μ M IBMX and 10 μ M FSK for the final two minutes of the acquisition. GFP fluorescence was quantified from whole-islet ROIs using Fiji software (v1.54i), and mean intensities were obtained for each region of interest (ROI) and time point. Results were presented relative to the average fluorescence intensity per islet during the 6mM glucose baseline phase prior to agonist addition.

2.3.6 Islet immunofluorescence

Islets from *hGlp1r*^{A316T/A316T} or *hGlp1r*^{+/+} littermates (5-10 islets per condition) were fixed in 4% PFA, washed and permeabilized in PBS containing 0.5% (v/v) Triton-X-100 for 15 minutes. Islets were then blocked in PBS containing 1% BSA for 1 hour, before incubating in primary antibodies against insulin (1:50, IR002, Dako, Agilent, USA) and glucagon (1:500; G2654, Sigma-Aldrich, UK) overnight at 4°C. After three washes with PBS, islets were incubated for 1 hour at room temperature with secondary anti-guinea pig Alexa Fluor 488 (1:500, ThermoFisher Scientific) and anti-mouse Alexa Fluor 647 (1:500, ThermoFisher Scientific), to visualise insulin and glucagon positive cells in islets, respectively. Islets were then loaded onto glass-bottom dishes and imaged in PBS, acquiring three images at different z-stacks by confocal microscopy with an Imperial College London NHLI FILM Zeiss LSM-780 inverted confocal laser scanning microscope and a 63x/1.4 numerical aperture oil immersion objective. Images were then analysed using Fiji (v1.53c).

2.3.7 High-resolution confocal imaging of hGLP-1R trafficking and surface expression in islets

High-resolution confocal imaging of hGLP-1R

Islets from *hGlp1r*^{A316T/A316T} or *hGlp1r*^{+/+} littermate controls (both chow and high-fat, high sucrose diet (HFHSD) fed conditions) were labelled with 1 μ M exendin-9-TMR (a fluorescent GLP-1R antagonist) in complete medium at 37°C for 10 minutes, to visualise endogenous hGLP-1R plasma membrane expression. Islets were then washed briefly in PBS, loaded onto glass-bottom dishes, and images were acquired in PBS by confocal microscopy with an Imperial College London NHLI Zeiss LSM-780 inverted confocal laser-scanning microscope and a 63x/1.4 numerical aperture oil immersion objective. Images were acquired for islets from 5 different animals per condition and were then analysed using Fiji v1.54i software. Islets from *hGlp1r*^{-/-} transduced with pAV-CMV-SNAP-*hGlp1r*^{WT} or *hGlp1r*^{A316T} adenoviruses were labelled with SNAP-Surface 647 (New England Biolabs, UK) at 37°C for 10 minutes. Post-labelling, the processing and imaging for these islets was done as mentioned above.

Lysosomal inhibition in islets

The same mouse islets mentioned above were incubated for 2 hours in complete medium with or without bafilomycin-A1 (100 nmol/L, 400 μ M, Sigma-Aldrich, UK), an established vacuolar H⁺-ATPase and lysosomal trafficking inhibitor. Islets were then labelled and imaged as before to assess receptor cell surface expression before and after lysosomal inhibition.

2.3.8 RNA extraction from islets

Total RNA was extracted from 50-100 islets isolated from *hGlp1r^{A316T/A316T}* mice and their *hGlp1r^{+/+}* littermate controls by incubating with TRIzol reagent (Invitrogen) for 10 minutes at room temperature, and briefly vortexed for homogenization. Chloroform (0.2 volumes, Sigma-Aldrich) was then added and the mixture briefly vortexed before centrifugation at 12,000 x g for 15 minutes at 4°C to achieve phase separation. Out of the three phases, the clear upper aqueous phase containing the RNA was collected into a clean 1.5 ml microcentrifuge tube. RNA was then recovered overnight by precipitation with isopropanol (0.8 volumes) ThermoFisher Scientific, Waltham, USA) followed by centrifugation at 12,000 x g for 5 minutes to precipitate the RNA into a pellet. Subsequently, the pellet was washed twice with 70% ethanol and resuspended in RNase free water. RNA concentration was then quantified using Nanodrop Spectrophotometer (ThermoFisher Scientific).

2.3.9 Reverse transcription (cDNA synthesis)

RNA (100-500ng) was reverse transcribed using the MultiScribe Reverse Transcriptase (ThermoFisher Scientific, UK) according to the manufacturer's instructions.

2.3.10 Quantitative PCR (qPCR) for detection of mRNA

Quantitative PCR (qPCR) was performed using a SYBR Green master mix on an Applied Biosystems 7500 real-time PCR system. β -actin gene expression was used as endogenous

control. Data were analysed using the $2^{-\Delta\Delta C_t}$ method (534). A list of qPCR primer sequences is provided in Table 2.4.

Table 2.4 Primer sequences used for qPCR in this PhD project.

Gene	Forward sequence (5' → 3')	Reverse sequence (5' → 3')
β-ACTIN	CACTGTCGAGTCGCGTCC	TCATCCATGGCGAACTGGTG
MAFA	CTTCAGCAAGGAGGAGGTCATC	CGTAGCCGCGGTTCTTGA
ACOT7	AGATGATTGAGGAGGCCGG	ACAGCGCTCCCCATTCTG
LDHA	ATGAAGGACTTGGCGGATGA	ATCTCGCCCTTGAGTTTGTCTT
SLC16A1	GCTTGGTGACCATTGTGGAAT	CCCAGTACGTGTATTGTAGTCTCCAT
GCG	CCAAGAGGAACCGGAACAAC	CCTTCAGCATGCCTCTCAAAT
INS1	GCTGGTGGGCATCCAGTAA	AATGACCTGCTTGCTGATGGT
KCNJ11	CACGGCGGGATAAGTCTACCT	AATCATTTGCCCCCTTCTTGT

2.4 *In vivo* experiments

2.4.1 Animal maintenance and diet

All animal procedures were approved by the British Home Office under the UK animals (Scientific Procedures) Act 1986 (Project License number PP7151519 to Dr Aida Martinez-Sanchez, Imperial College London, UK) and from the local ethical committee (Animal Welfare and Ethics Review Board) at the Central Biological Services unit of Imperial College London. Animals were housed in groups of up to four adult mice in pathogen-free individually ventilated cages with 12:12 light to dark cycles (lights on at 7:00) and under controlled temperature and humidity conditions between at 21-23°C and 45 and 65%, respectively. Ad libitum access to deionised water and feed was provided unless otherwise required for experiments. Lean mice were fed a standard chow diet (RM1 (E); Special Diet Services, UK). For high-fat, high sucrose diet (HFHSD) studies, animals were put on a 58% kcal fat and sucrose diet (D12331, Research Diet, New Brunswick, NJ) ad libitum for 12-14 weeks prior

to experiments. At the end of *in vivo* studies mice were sacrificed using cervical dislocation; the same methods of euthanasia were applied to ailing animals. Mice were weighed each before and each week from the time HFHSD feeding started to determine changes in weight gain for each genotype.

2.4.2 Generation of *hGlp1r*^{A316T/A316T} mutant mice

Transgenic mice were generated on a C57BL/6 *hGlp1r*^{+/+} mouse background from Taconic Artemis GmbH that has been described before (549). These mice express the *hGlp1r* from the murine *Glp1r* promoter and endogenous upstream regulatory elements. Whereby, exon 2 of the *hGlp1r* is placed immediately after the *mGlp1r* 5' UTR, start codon, and signal peptide sequences, generating a 100% mature human GLP-1R protein with a C-terminal FLAG epitope upon proteolytic cleavage and transport to the plasma membrane (Supplementary Figure 1A). The C-terminal flag epitope enables the use of anti-FLAG antibodies, immunoblotting and affinity purification. Moreover, the *hGlp1r* is flanked by LoxP sites that allow conditional deletion of *hGlp1r* using Cre recombinase, for example these animals can be crossed with *Rosa26Cre* transgenic mice to generate whole-body *hGlp1r*^{-/-} mice.

To generate mouse embryos with the *hGlp1r*^{A316T/A316T} genotype, the TAKE method was used to introduce the Cas9 nuclease, single-guide RNA (gRNA) and donor single-stranded oligo DNA nucleotides (ssODN) into intact *hGlp1r*^{+/+} mouse embryos using a super electroporator NEPA 21 (NEPA GENE Co. Ltd., Chiba, Japan). Briefly, pronuclear-stage embryos were placed in a line on the glass chamber between 5mm gap platinum plates (CUY520P5, NEPA GENE Co. Ltd.) that were filled with 100 µL Opti-MEM medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 1.2 µM Alt-R Cas9 Nuclease 3NLS (IDT, cat# 1074181), 6

uM gRNA and 300 ng/ μ l ssODN. The poring pulse was set to voltage: 225 V, pulse width: 2 mseconds, pulse interval: 50 mseconds, and number of pulses: 4. The first and second transfer pulse were set to voltage: 20 V, pulse width: 50 msecond, pulse interval: 50 msecond and number of pulses: 5. After electroporation, all embryos were cultured before embryo transfer. Embryos that developed to the two-cell stage after introduction of ribonucleoprotein (RNP) and ssODN were transferred into the oviducts of female *hGlp1r*^{+/+} surrogates that have been anesthetized using isoflurane. Surrogate females were then mated with vasectomized *hGlp1r*^{+/+} male mice a day prior to embryo transfer.

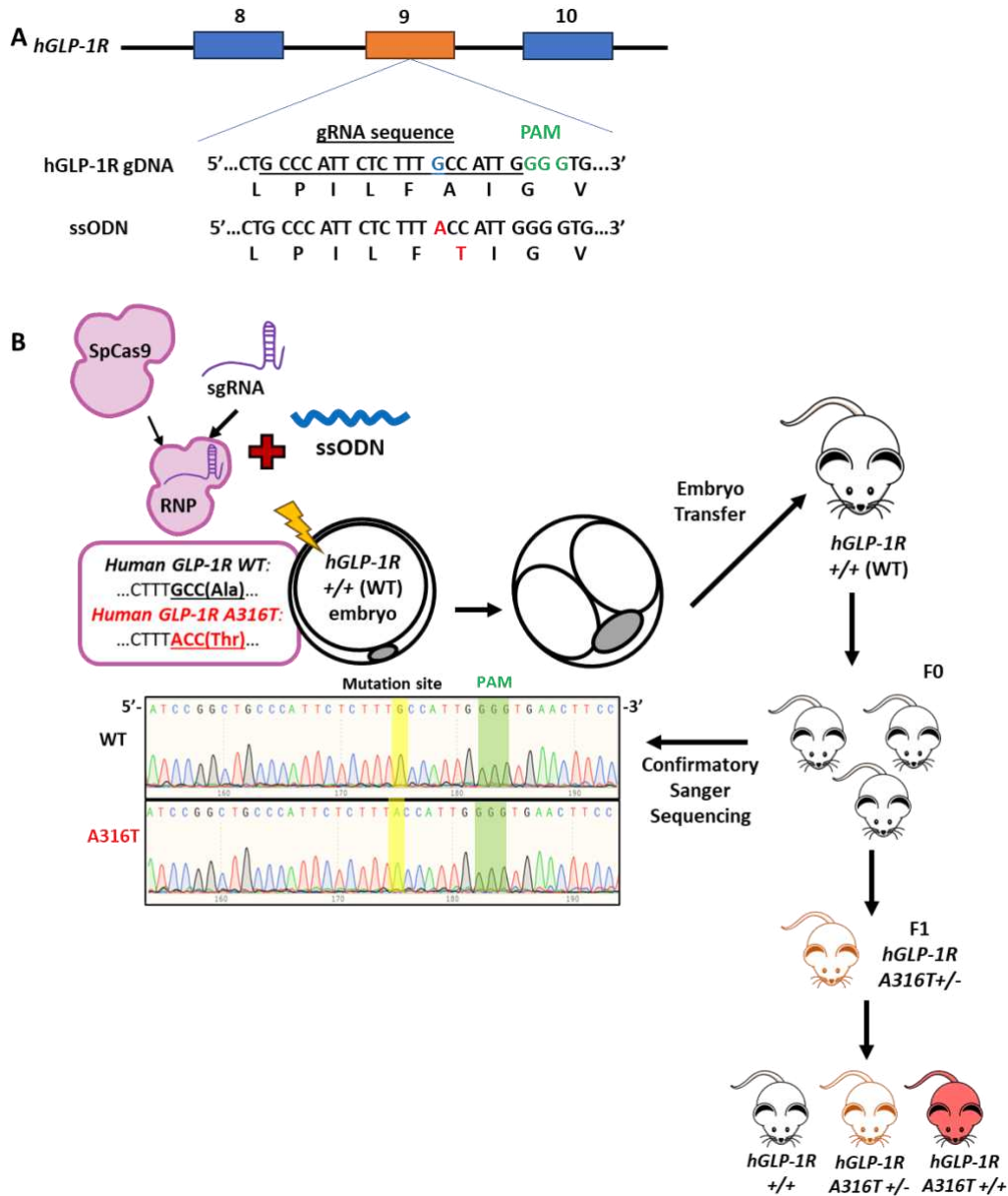


Figure 2.9 Generation of *hGlp1r*^{A316T/A316T} mice by CRISPR/Cas9. (A) Schematic diagram showing the strategy to generate the *hGlp1r*^{A316T/A316T} knock-in mice. Targeting strategy for the generation of *hGlp1r*^{A316T/A316T} knock-in mice using CRISPR single guide RNA (sgRNA) to target the Cas9 nuclease to the GCC region of exon 9 in the human GLP-1R gene and the corresponding ssODN homology directed repair template. (B) Schematic diagram of the electroporation of Cas9 nuclease, sgRNA and ssODN templates into the pronucleus, followed by transfer of two cell embryo into *hGlp1r*^{+/+} female mice resulting in the F0 generation. The screening strategy of F0 mice and further generations for experiments via genomic PCR followed by NGS sequencing. Sequences of *hGlp1r*^{+/+} (WT) and *hGlp1r*^{A316T/A316T} (A316T) mice.

DNA nucleotides, Sp. Cas9 Nuclease and sgRNA were purchased from Integrated DNA Technologies (IDT, Coralville, Iowa, US). The sgRNA used to generate the Alanine to Threonine mutation was designed using the RGEN tool (<http://www.rgenome.net/cas-designer/>) using a target sequence. The ssODNs were designed using the Alt-R CRISPR HDR design tool (<https://eu.idtdna.com/pages/tools/alt-r-crispr-hdr-design-tool/>), using the same target sequence as for the sgRNA (Figure 2.9). The sgRNA sequence used and its corresponding ssODN sequences are listed in Table 2.5.

Table 2.5 sgRNA and corresponding ssODN oligonucleotide sequences utilised for the CRISPR/Cas9 genome editing approach used to generate the *hGlp1r*^{A316T/A316T} mice

Type	Sequence
sgRNA	5'- GCCCATTCTCTTTGCCATTG-3'
ssODN Forward	5'- CATGAACTACTGGCTCATTATCCGGCTGCCCATTTCTCTTTACCATTGGGGT GAACTTCCTCATCTTTGTTCGGGTCATCTGCAT-3'
ssODN Reverse	3'- ATGCAGATGACCCGAACAAAGATGAGGAAGTTCACCCCAATGGTAAAGA GAATGGGCAGCCGGATAATGAGCCAGTAGTTCATG-5'

2.4.3 Genomic DNA extraction and genotyping

To genotype the transgenic mice, ear samples were first collected from weaned animals. Isolation of mouse DNA for genotyping was then performed in an alkaline lysis buffer [25 mM NaOH and 0.2 mM EDTA (pH 8.0) in deionised H₂O] for 1 hour at 95°C and subsequently neutralised by the addition of 13 mM Tris-HCl (pH 7.4) (362). Purified genomic DNA was then used to generate PCR templates for Sanger sequencing. PCR was performed in a total volume of 25 µL under the following conditions: 1 cycle of 94°C for 5 minutes; 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 68°C for 60 seconds; and 1 cycle of 68°C for 5 minutes. The final reaction mixture contained 10 µL Taq 2x Master Mix (New England

Biolabs), 9.5 µL Nuclease-free water, 2 µL genomic DNA (gDNA), and 0.2 µM of each primer (0.5 µL) amplifying ~500 bp locus flanking the single nucleotide polymorphism. The PCR products were then sent for Sanger sequencing at GeneWiz/Azenta, UK. The primers used for genotyping and sequencing are described below in Table 2.6.

Table 2.6 Primer sequences used for genotyping and Sanger sequencing of *hGlp1r*^{A316T/A316T} mice

Primer Name	Sequence
Outer Forward Primer	5'- GCAGTACTGTGTGTGGCGGCAATTACTACT- 3'
Outer reverse Primer	3'- GTCCATCACAAAGGCAAAGATGACCTCA-5'
Sequencing Primer (A316T-SEQ)	5'- GTACCTGTACACACTGCTGG-3'

2.4.4 Oral Glucose Tolerance Test (OGTT)

Lean or HFHSD fed *hGlp1r*^{A316T/A316T} and littermate controls were fasted 5 hours prior to the experiments. A glucose challenge (2 g/kg) of body weight was administered into the gut by oral gavage. Blood glucose was then serially recorded at 0 (before glucose administration), 10, 30, and 60 minutes after glucose administration via tail venepuncture using an automatic glucometer (Accu-Check, Roche Burgess Hill, UK).

2.4.5 Intraperitoneal Glucose Tolerance Tests (IPGTTs)

To examine acute and prolonged glycaemic responses to GLP-1R agonists, two separate IPGTT experiments were performed on the same day: acute and long term. IPGTTs were

carried out using a crossover design to administer vehicle or different GLP-1RAs to the same animals, with each animal receiving each treatment at least 3 days apart. This approach enables the calculation of delta AUCs (areas under the curve) for each agonist in the same animals, reducing the impact of individual animal differences on the observed treatment effects. Animals were fasted for 2 hours (at 8 a.m.) prior to the performance of the first acute IPGTT. As with OGTTs baseline glucose readings were taken at time point 0 via tail venepuncture and 10, 30, and 60 minutes after glucose $\pm$ agonist administration (2 nmol/kg exendin-4 and exendin-F1), using an automatic glucometer (Accu-Check, Roche Burgess Hill, UK). IPGTTs were repeated 6 hours after initial glucose $\pm$ agonist administration (long term), and after that the food was topped up. For GLP-1RAs with a longer half-life, IPGTTs were performed 24 and 72 hours after glucose $\pm$ 20 nmol/kg agonist (tirzepatide or semaglutide) administration, and food was topped at the end of each IPGTT.

2.4.6 Measurement of *in vivo* plasma insulin levels

Animals were fasted and administered glucose $\pm$ agonist corresponding to the IPGTT experiment. Blood from the tail vein was collected at 10 minutes post-agonist administration into potassium EDTA cuvettes (Microvette CB 300, 16.444.100, Starstedt) and stored on ice until centrifugation at 8,000xg for 10 minutes 4°C to collect plasma supernatant. Plasma was then collected into fresh Eppendorf tubes and kept at -80°C until analysis using the Revvity Mouse Plasma Insulin HTRF kit (62IN3PEF; Revvity, Waltham, Massachusetts, USA) following the manufacturer's recommended procedure. A total of 10 μ L of blood plasma sample was used to measure plasma insulin levels in duplicate.

2.4.7 *In vivo* pancreas hGLP-1R labelling

Animals were injected subcutaneously with 100 pmol/g of Luxendin645 and left for two hours before performing terminal anaesthesia (1.5% sodium pentobarbital) and transcardial perfusion fixation using phosphate buffer saline (PBS, pH7.2) and 4% fresh PFA (313). Pancreata were then isolated, weighed, cleared from all surrounding fat and non-pancreatic tissue, and washed twice in ice cold PBS. After washing, pancreata were post-fixed for 24 hours in 4% PFA at 4°C and then cryoprotected in 30% sucrose until the tissue sinks. When the pancreata sink, they were washed three times with PBS (5 minutes each) and embedded in optical cutting temperature (OCT) and stored at -80°C. Frozen 10µm sections were then prepared using a Bright OTF5000 cryostat, yielding 10 sections per animal per genotype and mounted on Superfrost Plus slides (ThermoFisher Scientific) and stored in a slide box in the -80°C until immunostaining.

For the measurement of endogenous GLP-1R expression in frozen pancreatic tissues, slides were defrosted, washed three times with PBS and mounted with Prolong Diamond Antifade Mountant with DAPI (P36962, ThermoFisher Scientific, UK). Images of sections were captured using a Nikon Eclipse Ti spinning disk confocal microscope and 60x oil immersion objective with an ORCA-Flash 4.0 camera (Hamamatsu) and Metamorph software (Molecular Devices), with images collected after excitation at $\lambda = 660\text{nm}$ and analysed in Fiji software (v1.54i).

2.4.8 Immunostaining of frozen pancreatic tissue sections

For quantification of β - and α -cell mass, whole pancreata were dissected from mutant and control animals, placed in ice cold PBS, before being processed as above. Frozen pancreatic tissue sections were then defrosted for 5 minutes before using a hydrophobic barrier pap pen to create a water-repellent barrier around each of the tissue sections on the microscope slide. Tissue sections were then washed by gently pipetting 500 μ L PBS three times for 5 minutes each at room temperature before replacing with 500 μ L PBS + 0.2% Triton X-100 (PBS-T) for 15 minutes to permeabilise the tissue. Non-specific binding is then blocked by incubating the tissue sections in 2% BSA for 1 hour at room temperature. Subsequently, the sections were immunostained by incubating with 2% BSA and 0.1% Tween-20 and primary antibodies: guinea pig anti-insulin (1:100; Dako, IR002, Agilent Technologies) for β -cell detection and mouse anti-glucagon (1:500; G2654, Sigma-Aldrich) for α -cell detection, overnight at 4°C. Slices were then washed with wash buffer (PBS and 0.2% Tween) and visualized with secondary conjugated antibodies: Alexa Fluor 488 goat anti-guinea pig IgG (1:500; Invitrogen) and Alexa Fluor 568 goat anti-mouse IgG, (1:500; Invitrogen). Samples were then washed and mounted with Prolong Diamond Antifade Mountant with DAPI (P36962, ThermoFisher Scientific, UK) for nuclei counting and to visualise total cell numbers. Stained slides were imaged at a 4x objective for the quantification of total pancreatic area, and at 40x to quantify insulin and glucagon positive areas in pancreatic islets using a Wide-field Zeiss Axio Observer inverted microscope. Total pancreatic, insulin-, and glucagon-positive areas were quantified for each slide using the Fiji (ImageJ) software. β - and α -cell mass were then calculated by normalising insulin- and glucagon-positive areas to total pancreatic section area

and pancreas weight as described before (550, 551). To ensure that only islet endocrine tissue was included in the analysis, non-islet regions were manually outlined and removed using the “Clear Outside” function in Fiji following selection with the freehand or polygon selection tool. Islet boundaries were readily identifiable due to the clear round morphology and strong glucagon staining, which enabled confident exclusion of non-islet insulin positive regions that may arise due to background insulin staining. This step excluded exocrine pancreas tissue and any signal outside the defined islet boundary. Within the cleaned islet regions, thresholding was applied to define insulin and glucagon signals for quantification. Islet size and cytoarchitecture analyses were performed on the same images used for β - and α -cell mass assessment. Cells were assigned to be in the islet periphery if they were within the two outermost cell layers of the islet, or in the islet core if they fell within any layers deeper than these. This classification enabled the assessment of spatial distribution patterns of α - and β -cells.

2.5 *In silico* experiments

The structural biology methods in this subchapter were entirely the work of our collaborators Dr. Kieran Deane-Adler supervised by Prof. Denise Wootten at Monash University, who resolved and determined the cryo-EM structure of the GLP-1: GLP-1R(A316T): $G\alpha_s$ and Ex-D3: GLP-1R(A316T): $G\alpha_s$ complexes.

2.5.1 Cryo-EM Determination of the GLP-1: GLP-1R(A316T): Gα_s complex

Construct Design

A human GLP-1R construct with residue Ala316 substituted for Thr (c.946G>A) was generated from the wildtype sequence by Q5 enzyme site-directed mutagenesis (New England Biolabs, USA) and cloned into the pFastBac vector. The native signal peptide was replaced with HA to enhance expression, and an N-terminal FLAG epitope and a C-terminal 6×His epitope were added as affinity tags with 3C protease cleavage sites. These modifications do not significantly alter the function of WT GLP-1R (284).

Purification of GLP-1/Exendin-D3: GLP-1R(A316T):DN-Gα_s:nb35

A-FLAG-GLP-1R(A316T)-His, DN-Gα_s, and Gβ1γ2-His P2 baculoviruses were co-infected in a 500 mL culture of T. ni Hi5 cells in suspension (density $3-4 \times 10^6$ cells/mL), at a ratio of 2:1:1. After 48 hours, the cell preparations were harvested by centrifugation at 8,000 g for 10 minutes and the supernatant discarded. The pelleted cell mass was stored at -80 °C until use.

For purification, cell preparations were rapidly thawed and resuspended in 50 mL of buffer. Receptor-G protein complex was formed in the presence of 10 μM GLP-1 for 30 minutes at room temperature with gentle stirring. After this period, nanobody 35 (1 mg) and apyrase (5 μL/L of starting culture, equivalent to 2.5 U) were added to the resuspension and incubated at room temperature for a further 30 minutes. Purified nb35 was a kind gift from Minakshi Baruah.

Vitrification and data collection of purified samples

2 μL of sample was applied to glow-discharged R1.1/1.2 gold foil grids, manufactured by Quantifoil and the Melbourne Centre for Nanofabrication (MCN) immediately followed by plunge-freezing in liquid ethane using a Vitrobot Mark IV (Thermo Scientific, USA) set at 4 $^{\circ}\text{C}$, 100% humidity. At least three grids were prepared for each sample, and blot force and blot time varied between 16-18 and 6-8 seconds, respectively. Vitrification conditions for grids used in data collection were as follows, blot force 16 and blot time 7 seconds. cryo-EM data were collected on a Glacios cryo-TEM (Thermo Scientific, USA) operating at 200 kV accelerating voltage in cryo-TEM nanoprobe mode with a 100 μm objective aperture inserted. Data were collected using aberration-free image shift in fast acquisition mode in the EPU software (Thermo Scientific, USA).

Data processing for GLP-1: GLP-1R(A316T): DN-G α s: nb35

3,170 micrographs were assigned to 69 optics groups based on their beam-image shift coordinates, motion corrected in RELION 3.1.2 and CTF parameters were estimated using CTFFIND 4.1 within RELION (552). For quality control, micrographs with an estimated maximum resolution >20 $\text{\AA}$ were excluded, leaving 2940 micrographs in the dataset. 1,667,111 particles were picked using a pre-trained general model in crYOLO 1.7.6 (553) and extracted with 4.5-fold binning. These particles were imported into cryoSPARC 3.2 (554) and subjected to rounds of 2D classification to homogenise the particle stack, with 583,410 particles remaining. These particles were re-extracted at the full box size (288 px²) and subjected to Bayesian polishing in RELION 3.1.2. The polished particles were re-imported into cryoSPARC, filtered for polishing artefacts by 2D classification, and a final set of 330,000

was subjected to non-uniform refinement to obtain a 3.3 Å global resolution consensus density map.

Atomic model refinement used an existing model of wild-type GLP-1R bound to GLP-1/Exendin-D3 and DN-G α_s proteins (PDB: 6X18); the alanine at position 316 of GLP-1R was mutated to threonine. The mutated model was fitted into the experimental density map and subjected to MDFF refinement using Isolde (555) within ChimeraX 1.5 (556). Iterative rounds of real-space refinement in PHENIX 1.20 (557), followed by MDFF in Isolde, were used to obtain the final model for analysis.

2.5.2 Computation methods and MD simulations

The MD simulations and computational methods in this subchapter were entirely the work of our collaborators Dr. Roxana-Maria Rujan supervised by at Coventry University.

GLP-1R WT and A316T inactive structure MD simulations

The inactive GLP-1R structure, solved by Wu *et al.* (2020) was retrieved from the Protein Data Bank (PDB 6LN2) (295), then missing residues in the stalk (129-134) and ICL2 (258-261) regions were modelled using Modeller 9.18 (558) through Chimera (556) (v1.14) user Interface, considering the best DOPE score. The A316T variant receptor was then constructed by employing PyMOL (The PyMOL Molecular Graphics System, Version 3.0 Schrödinger,

LLC) to substitute alanine at position 316 into threonine in the GLP-1R WT TM5 region. The resulting GLP-1R TMD systems (i.e., WT and A316T) were prepared for MD simulations, after superimposition with the PDB 6LN2 OPM reference (559), according to the protocol described previously in Zhao *et al.* (2020) (560). The inactive WT receptor was simulated for a total of 8 μ second in 4 replicas of 2 μ second, while the inactive A316T receptor variant was simulated for 3.5 μ second in 7 replicas of 500 seconds.

GLP-1R WT and A316T active structure MD Simulations

To perform simulations on active GLP-1R A316T bound to GLP-1, an already patched and minimized structure based on a GLP-1R:GLP-1: $G\alpha_s$ protein cryo-EM structure, solved by Zhang *et al.* (PDB:6X18), was used. For the GLP-1R A316T bound to exendin-4, a patched and minimized structure based on an earlier version of PDB:7LLL, a GLP-1R:Exendin-4: $G\alpha_s$ protein cryo-EM complex solved by Deganutti *et al.* (2022) was used (374). The starting point for simulations of WT GLP-1R simulations bound to either Ex-D1 or Exendin-D3 were unpublished cryo-EM structures obtained from our collaborators Prof. Denise Wootten and Prof. Patrick Sexton at Monash University. As before using these structures as a starting point the alanine position at position 316T was substituted for threonine to obtain the A316T variant receptor, using PyMOL (The PyMOL Molecular Graphics System, Version 3.0 Schrödinger, LLC). The MD protocol steps were described previously as above. Concerning simulations of the WT receptor, simulations computed and published before were used for the GLP-1R: GLP-1: $G\alpha_s$ complex, and unpublished simulations were used for the GLP-1R: exendin-4: $G\alpha_s$ complex. The total time of simulations for each GLP-1R WT system was 2 μ seconds in 4 replicas of 500 nseconds, and 3 μ seconds in 6 replicas of 500 nseconds for each A316T system coupled to either GLP-1 or exendin-4 peptides and $G\alpha_s$ protein.

MD analysis

Every replica of each system was merged using mdconvert from MDTraj (561). The Root Mean Square Fluctuation (RMSF) was computed using VMD (Humphrey, et al., 1996) on Ca atoms 140 to 402 for each receptor (active and inactive). Distances between residues were computed using the open-source community-developed library PLUMED 2.0 (www.plumed.org), while the data was plotted using the Python package, Seaborn (Waskom, 2021). Polar interactions and contact interactions occurring between residues within the receptor, ligand-receptor and receptor-G-protein were quantified using the GetContacts analysis tool available at <https://getcontacts.github.io/> and quantified as the percentage of frames (over all the frames obtained by merging the different replicas) in which they were present. Hydrogen bonds obtained were of side chain-side chain (HBSS), backbone-side chain (HBSB) and backbone-backbone (HBBB) subtypes. Water molecule occupancy was computed employing the VMD Volmap plugin (<https://www.ks.uiuc.edu/Research/vmd/plugins/volmapgui/>) with 1 Å resolution (548). The difference in interactions were plotted on structures using UCSF Chimera (Pettersen *et al.*, 2004). Any other structure representation was conducted in PyMOL (www.pymol.org).

Numbering system

Throughout the manuscript, the Wootten residues numbering system for class B GPCRs (562) is adopted.

2.6 Statistical analyses

All data analyses and graph generation were performed with GraphPad Prism 10.1.2. The n numbers refer to the number of independent biological replicates, this represents the number of mice per genotype for *in vivo* experiments, or the number of biologically independent experiments performed from islets isolated from separate mice or a separate batch of cells, for *ex vivo* or *in vitro* experiments, respectively. Technical replicates within the same assay were averaged to determine the mean value for each biological replicate, prior to statistical tests. Statistical tests and n numbers are indicated in the corresponding figure legends. Statistical significance was assessed using student's two tailed t-test or ANOVA as indicated, with matched analyses performed for matched design experiments. Statistical significance was inferred when $p < 0.05$. Unless indicated, values represented are the mean $\pm$ SEM.

Chapter 3: *In silico* and structural analysis

predict the effects of the rs10305492 variant

3.1 Introduction

Advances in cryo-EM have assisted in the discovery of many inactive and active GLP-1R structures in complex with various agonists such as semaglutide, tirzepatide, exendin-4, the allosteric modulator compound 2, non-peptide agonists and GLP-1 (293). These structures capture the subtle conformational changes promoted by differential ligand binding and provide a framework to understand mechanisms of GLP-1R activation. For instance, recent cryo-EM structures of the GLP-1R complexed with orally deliverable non-peptide agonists identified novel binding pockets, unique activation profiles, and active-state conformations induced by these agonists (563).

Interestingly, these studies also revealed the importance of structural differences involving water-mediated hydrogen networks stabilising agonist binding, especially correlated to functional data to show that oral small molecule non-peptide agonists such as danoglipron can closely resemble GLP-1 (287). Structural studies and resolved GLP-1R structures at high resolution continue to aid our understanding of intricate phenomena such as biased signalling; for instance, modest structural differences have already been revealed between GLP-1 and the biased exendin-4 analogue, exendin-P5 bound to GLP-1R- G protein complexes (384). Despite the rapid growth in GLP-1R structure elucidation, these structures lack the highly variable and

dynamic regions in the extracellular and intracellular loops, and most terminal domains, which are modelled *in silico* for structural studies (293, 295, 563-565).

Advanced sequencing methods have increased the identification of nonsynonymous missense mutations. In the era of precision medicine, understanding the role of natural variants on GPCR function, is critical from a pharmacogenomics point of view. A large number of variants is observed in the *hGlp1r*, as listed in GPCRdb and the gnomAD databases, with an average of ~340 missense variants noted for the receptor (566, 567). Of these variants, some are clinically and physiologically significant, and they are classified into GoF and LoF mutations, likely to alter receptor structure resulting in altered ligand recognition, binding affinity, and G protein specificity, and result in dysregulation of receptor membrane localisation, signalling and function.

Resolving GPCR structures and *in silico* mapping is commonly the first step taken to understand the functional consequences of GPCRs lacking structural information, such as GPCR variants, followed by MD simulations to elucidate alterations in mutant receptor dynamics, aiding in variant-specific ligand design. In addition, this variant specific structural analysis can be correlated to the functional effect of mutations in experimental studies (568, 569). MD simulations serve as a virtual lens, enabling detailed examination of GPCR behaviour at an atomic level (570). Mutations at specific residues can trigger disturbances that may be local or extend over a larger range, compared to the normal, wild-type structure. GPCRs operate through interconnected atomic motions, with or without the presence of a ligand, and the functional dynamics of a mutant receptor could differ from those of the wild-type receptor (570-572). MD simulations offer a way to comprehend and visualise these alterations in movement with spatial and temporal precision. A detailed knowledge of active

and inactive wild-type structures and dynamic conformations in the presence of various ligands is essential as a starting point for comparison with mutant GPCR structures, thus aiding to understand the role of distinct SNPs.

So far, studies have used structural analysis including cryo-EM structures and MD simulations to predict facets of receptor function affected as a result of variants in GPCRs such as the β_2 -adrenergic receptor (β_2 AR) (569), cannabinoid receptor 1 (CB1) (573), gonadotropin-releasing hormone receptor (GnRHR), kisspeptin receptor (KISS1R), prokineticin receptor (PROKR2), tachykinin receptor 3 (TACR3) (574) and glucagon receptor (GCGR) (509, 511). Some of these variants were identified in patients with disorders and have then been studied *in vitro* and clinically, which has aided in the interpretation of the correlation between genotype and phenotype.

On the other hand, structural and *in silico* information for GLP-1R variants has not been well established. Wessel *et al.* (2105) tried to predict the functional consequences of the GLP-1R A316T variant by modelling the mutant receptor based on the published full-length exendin-4-bound human GLP-1R structure by MD simulations on this model (43). MD results suggested alteration of hydrogen bonds between residues in TM5 (N320) and TM6 (E364), altering the conformations of ECL3 and TM6, potentially affecting G protein recruitment and downstream signalling. In parallel to this, a recent paper by Lagou *et al.* (2023), also performed MD simulations to gain structural insights into GLP-1R variants including A316T, G186S, and R421W (518). However, unlike the previous study, here they used a human wild-type GLP-1R structure bound to oxyntomodulin as a starting point. Their results showed that A316T and G186S mutants alter the nearby hydrogen bond network that normally stabilises the GLP-1R inactive state; for A316T the T316 replaces Y242 in a persistent hydrogen bond

with P312 triggering conformational rearrangements in TM3 and 5, and enhancing G protein coupling; in G186S, a new hydrogen bond is formed between residues S168 and A164 moving the TM1 closer to TM2 and reducing overall flexibility of ICL1 and altering G protein activation.

In this chapter, I report further structural bioinformatic studies of the GLP-1R A316T variant. The experiments I discuss here were carried out by our collaborators Dr Roxana Maria Rojan, Dr Guiseppe Deganutti, and Prof Christopher Reynolds in the Centre for Sports, Exercise and Life Sciences at Coventry University. They include MD simulations of the inactive- and active-state structures of the GLP-1R bound to GLP-1, exendin-4, and its analogues exendin-F1 and exendin-D3. In addition to this, I also discuss additional structural insights obtained by our collaborators Dr Kieran Deane-Adler and Prof Denise Wootten at the Monash Institute of Pharmaceutical Sciences, Australia, including resolved high resolution cryo-EM structures of the GLP-1R A316T variant in complex with GLP-1R agonists (GLP-1 and exendin-D3) as well as $G\alpha_s$.

3.2 Results

3.2.1 MD simulations: Impact of the A316T variant on residue-residue interactions, hydrogen bond formation and receptor dynamics on the inactive (apo-state) GLP-1R

As there are key differences between peptide-free and peptide-bound GLP-1R states, our collaborators evaluated the differences between the WT and A316T GLP-1R peptide-free inactive state structures. As mentioned before, MD simulations have been utilised to study the conformational dynamics of GLP-1R and have provided valuable information regarding the molecular basis of receptor dynamics and ECD transitions between the inactive and active states. Hence, our collaborators performed MD simulations to compare intermolecular contacts and hydrogen bond interactions between the inactive WT and A316T GLP-1R structures using the inactive WT GLP-1R structure, PDB 6LN2 solved by Wu *et al.* (2020), as a starting point to model the mutant receptor. They found that the inactive state A316T receptor exhibits fewer intramolecular contacts compared to the WT receptor, but more intramolecular interactions in regions such as ECL1, and ECL3/TM7 (Figure 3.1A). In terms of hydrogen bond interactions, substituting the alanine for threonine in the 316 position led to the formation of a weak hydrogen with N320^{5.50} and two additional persistent bonds with the backbone of P312^{5.42}, and with the side chain of Y242^{3.45}, disrupting a naturally formed hydrogen bond between these two residues present in the WT receptor (Figure 3.1B and C). In order to find out the contribution of the mutant T316 residue on receptor dynamics in the absence of ligands, trajectories were analysed by calculating the RMSF of the inactive WT and A316T structures. These measurements revealed that the mutant receptor has less flexibility in the ECD, TM1

and top of the TM6 during simulations (Figure 3.1D). Conversely, the N-terminal helix of the ECD, top of TM2 and ECL1 regions were highly dynamic in the mutant receptor. Interestingly, comparison of the RMSF data of the active GLP-1: WT GLP-1R structure with the inactive A316T receptor revealed that the inactive state A316T receptor adopts an ECD conformation which is an intermediate state between the active and inactive GLP-1R WT (Figure 3.1D).

Next, our collaborators wanted to elucidate whether ECD flexibility of the inactive A316T receptor correlates with closer proximity between the TM6 and TM7 domains, as observations from the simulations showed differences in conformations adopted within these regions. Therefore, they analysed the distance between residues H363^{6.52} (near the TM6 kink) and F390^{7.45} in TM7 (Figure 3.1F). In the inactive GLP-1R (WT), this distance was diffuse, indicating a highly flexible ECD, whereas the A316T mutant exhibited intermediate flexibility with a more consistent distance between TM6 and TM7 (Figure 3.1E and G). The distance between T51^{ECD} (last residue of the NTER helix in the ECD) and S301^{ECL2} was also measured, revealing that the WT receptor had shorter distances, while the A316T mutant showed longer distances, correlating with wider openings between ECD and ECL2. On the other hand, no significant changes were observed in the distance between the distal sides of TM6 and TM7. In the activated WT GLP-1R, the bottom part of TM6 moves outward, with slight conformational changes in TM7 and TM1, while the top parts of TM6 and TM7 come closer and interact more strongly (Figure 3.1E). This analysis suggests that the A316T mutation promotes an intermediate active-like conformation with closer TM6 and TM7 at the top, reducing ECD flexibility and enhancing its openness for peptide binding and interaction.

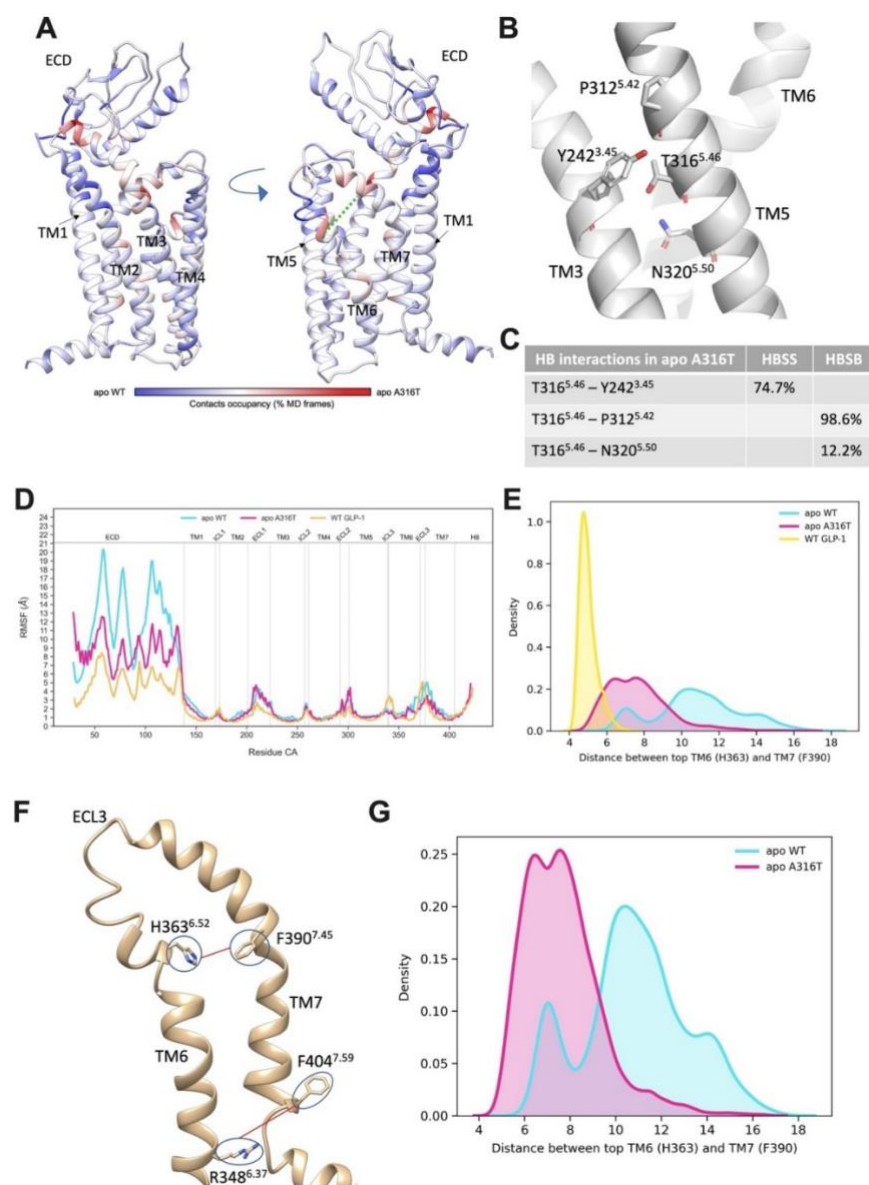


Figure 3.1 Impact of the A316T polymorphism on intramolecular contracts, hydrogen bond interactions and GLP-1R dynamics in the absence of agonists. A) Receptor intramolecular contacts taking place in apoform receptors during simulations. Residues in blue form more contacts in the apoform WT receptor, while those in red are more prone to interact in the A316T receptor. B) Hydrogen bonds formed in the apoform A316T receptor. C) Graphical representation of the A316T receptor at the site of the mutation. D) RMSF of the inactive structures and the active GLP-1:GLP-1R (WT) complex. E) Density distribution plot of the distance between TM6 and TM7 for the inactive receptors and the active GLP-1: GLP-1R (WT) complex. F) Graphic representation of the distance between TM6 and TM7 for the residues chosen. G) Same as E but without the data for the active GLP-1R WT bound to GLP-1 added.

3.2.2 MD simulations: Impact of the variant on receptor dynamics, residue-residue interactions, and peptide-receptor interactions of the ligand activated GLP-1R.

To explore the intrinsic dynamics of the ligand-bound A316T GLP-1R, our collaborators applied MD simulations to the crystal structures of modelled A316T with the GLP-1R agonists GLP-1, exendin-4 and its analogues exendin-F1 and exendin-D3 and $G\alpha_s$.

Firstly, they focused on the RMSF of $C\alpha$ atoms to understand the mutant receptor's flexibility with the different ligands bound. When the A316T GLP-1R is bound to GLP-1, it exhibits reduced flexibility in regions such as the extracellular domain (ECD), extracellular loop 1 (ECL1), and intracellular loop 3 (ICL3), whilst it exhibits increased flexibility of the TM6 kink, crucial for receptor activation (Figure 3.2A). In addition to this, some regions, particularly transmembrane domains (TM) 2 3,4 and 6, showed increased mobility. On the other hand, with the exogenous agonist exendin-4 bound, there was no notable differences in flexibility at the mutation site, but our collaborators observed increased dynamics in the ECD's stalk region, ECL1, and slight changes in TM3-5, like the GLP-1 bound structure (Figure 3.2B). Strikingly, the A316T mutation led to increased flexibility of the whole receptor when bound to the $G\alpha_s$ biased exendin-4 analogue exendin-F1, whereby marked modifications in receptor dynamics were seen in the ECD, TM1, TM3-5 and various loops (ECL1, ECL2, ECL3/TM7) regions (Figure 3.2C). Finally, binding of this mutant receptor to the other β -arrestin 2 biased exendin-4 analogue, exendin-D3, elicited similar alterations in dynamics to the exendin-4 bound structure, with no changes in the flexibility of the T316 residue, but changes in dynamics of the ECD stalk, ECL1-2, TM5 and the TM6/ICL3/TM7 region, as well as the CTER helix (Figure 3.2D). In conclusion, the A316T polymorphism in the GLP-1R

influences receptor dynamics differently depending on the ligand bound, which could underly differences in receptor function and signalling.

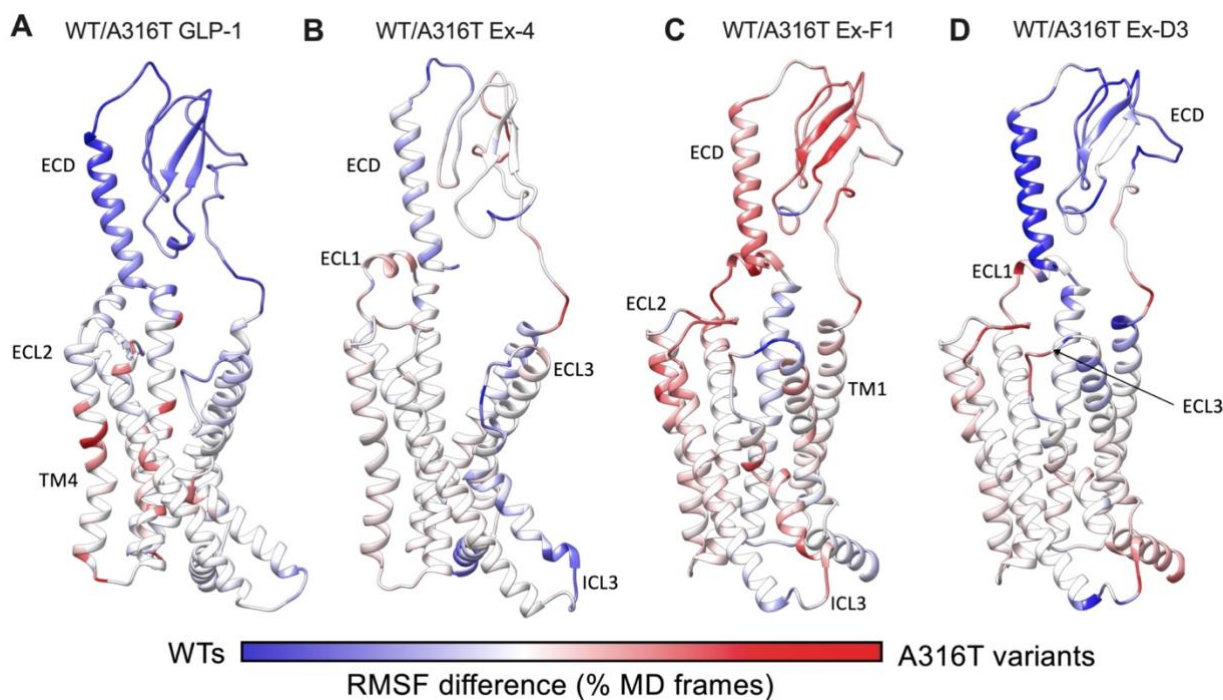


Figure 3.2 Differences in the RMSF of the C α atoms between the GLP-1R WT and A316T bound to GLP-1, exendin-4 (Ex-4), exendin-F1 (Ex-F1) or exendin-D3 (Ex-D3). A) GLP-1 B) Ex-4 C) Ex-F1 D) Ex-D3. Red colour indicates more dynamics in the A316T variant, while blue colour signals more flexibility in the WT receptor.

With regards to intra-receptor residue-residue interactions, the ligand bound A316T GLP-1R exhibits a similar pattern to the ligand-free A316T receptor, with the persistence of a bond between T316^{5.46} with P312^{5.42} and Y242^{3.45} (Table 3.1). However, whilst these interactions are present all the time in exendin-4 or exendin-4 analogue bound structures, it is only present half of the time in the GLP-1 bound structure. Moreover, T316^{5.46} shows stronger interactions with the backbone of I313^{5.43}, especially when bound to GLP-1 (Table 3.1). A316T structures bound to exendin-4 analogues exhibit strong interactions between N320^{5.50} near the TM6 kink and the backbone of L360^{6.49} and L244^{3.47}, akin to the WT receptor in complex to exendin-4.

Unlike the A316T GLP-1R, the WT GLP-1R exhibits stronger interactions with G361^{6,50}, L244^{3,47}, and L245^{3,48} in complex with the exendin-4 analogues exendin-F1 and -D3 (Table 3.1). Our collaborators think that these changes occurring at N320^{5,50} relate to differences in stabilisation of the TM6 kink related to receptor activation or partial activation and water mediated hydrogen bonds. A further test would involve looking at alanine substitution of N320^{5,50} in the A316T receptor in complex with the exendin-F1 and -D3, as the N320^{5,50} is involved in a hydrogen bonding network related to allosteric effects of G protein binding.

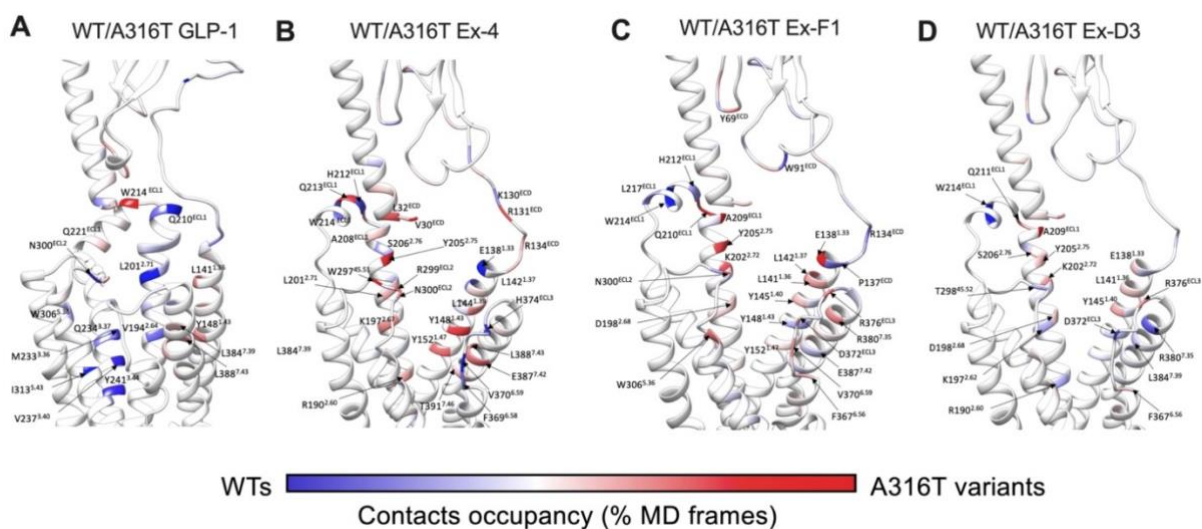


Figure 3.3 Peptide-receptor contact difference for the WT and mutant receptors bound to GLP-1, exendin-4 (Ex-4), exendin-F1 (Ex-F1) and exendin-D3 (Ex-D3). A) GLP-1 B) Ex-4 C) Ex-F1 D) Ex-D3. The red colour indicates receptor residues that interact more with the peptide in the variant, while blue colour indicates residues more prone to interact with the peptide in the WT.

In addition to this, hydrogen bond analysis revealed changes in peptide-receptor interactions between the WT and A316T receptor. The A316T polymorphism disrupted hydrogen bonds between H7 and R310^{5,40} and increased the interaction between E9 and R376^{ECL3} in the presence of GLP-1, yet all interactions were increased when bound to exendin-4 (Figure 3.3A and B). Although a similar pattern is seen in complex with exendin-F1, there is a 3-fold

increase in the interaction between D9 peptide residue and R376^{ECL3}, as well as rearrangements of interactions from T7 to ECL3, resulting in the ECL3 adopting a more open conformation and an increase in ECD flexibility (Figure 3.3C and D). One limitation of these structural computational studies is that they use modelled structures of the A316T, so the next steps would be to perform these simulations using the cryo-EM structure of the GLP-1R A316T.

Table 3.1 Changes in hydrogen bonds occupancy at and near the site of mutation in GLP-1R (WT) and (A316T) bound to the peptides. The occupancy of hydrogen bonds, expressed in percentages, shows how many simulation frames from a total of 100% exhibits the interaction of interest. Unless stated, the hydrogen bond is HBSS (side chain-side chain), while (bb) behind the residue represents the HBSB (backbone-side chain) type of interaction.

Residue	Residue	WT GLP-1	A316T GLP-1	WT Ex-4	A316T Ex-4	WT Ex-F1	A316T Ex-F1	WT Ex-D3	A316T Ex-D3
P312 (bb)	Y242	93.5	34.9	94.5	6.5	87.4	8.2	93.8	11
P312 (bb)	A/T316	-	55.6	-	90.7	-	88.8	-	83.7
N320	L360 (bb)	80.3	66.3	-	14.7	64.6	9.4	63.5	9.7
N320	L244 (bb)	76.4	54.6	19.5	14.8	61.2	21.3	68.5	31.4
N320	L245 (bb)	14.7	8.7	-	-	8.8	-	20	4.9
N320	G361(bb)	-	-	70.1	56.7	-	44.1	-	66.8
N320	Y241	-	-	-	11.1	-	-	-	-
I313 (bb)	A/T316	-	38.1	-	5.1	-	6.9	-	9.3
A/T316	Y242	-	26.7	-	54.7	-	42.4	-	41

3.2.3 Cryo-EM determination of the GLP-1: A316T GLP-1R: G α_s complex

To elucidate structural changes associated with the A316T variant receptor, the structure of the GLP-1 bound GLP-1R in complex with dominant negative (DN) G α_s was resolved to 3.3 Å by cryo-EM by our structural collaborators in Monash University. In general, the complex was well resolved with amino acid sidechains being reasonably modelled for almost the entire structure (Figure 3.4A). Due to the intrinsic flexibility of regions including the alpha-helical domain of (dominant negative) DN-G α_s , ECD, ECL3 and ICL3, they were more poorly defined. The overall positioning of the transmembrane domains (TMDs) and the binding arrangement of GLP-1, was extremely similar to the wild-type receptor previously reported by high-resolution cryo-EM (287) (Figure 3.4B). In the GLP-1R A316T similar to the GLP-1R WT, rich contacts along the inward face of the receptor TMDs guide the N-terminus of the GLP-1 deep into the orthosteric pocket, where it forms crucial peptide-receptor interactions with the conserved central polar network, including a salt bridge with R190^{2.60} and ECL1/2 (Figure 3.4C).

On the other hand, presence of the A316T substitution alters the network of structural waters connecting TM3,5 and 6, including hydrogen bonds likely formed with the highly conserved G361^{6.50} from the P^{6.47}xxG^{6.50} motif, which is crucial for the activation and downstream signalling of the receptor. In the GLP-1R (A316T): GLP-1, substitution of the A316^{5.46} to T316^{5.46} displaces the backbone

carbonyl of residue Y241^{3,44}, which in the GLP-1R WT directly interfaces with the central polar network via a hydrogen bond with a structural water located between TM3 and 5 (Figure 3.4D). This structural water is also capable of hydrogen bonding with the backbone carbonyl of A316^{5,42}, which may contribute to increased interhelical packing and stiffness. In the A316T variant, this backbone carbonyl is displaced around 1.5Å away from this structural water, probably reducing the interhelical packing and stiffness effect. Intriguingly, when modelled as the ‘upwards’ rotamer, the hydroxyl group of T316 could hydrogen bond with the oxygen of this structural water, but this rotamer position would likely preclude the known interactions discussed above.

Akin to peptide-receptor hydrogen bond interactions, A316T GLP-1R interactions with the $\alpha 5$ helix of $G\alpha_s$ are very similar to wild-type GLP-1R agonist-bound structures (Figure 3.4 E-G). Higher resolution structures of agonist-bound GLP-1R in complex with $G\alpha_s$ have uncovered crucial structural waters in this region, that act as a bridge between a highly conserved residue of the HETX motif (E247^{3,50}) and Y391 ^{$G\alpha_s$} . But at this resolution, these waters are not present in the density map, and the density of E247^{3,50} itself was quite poor compared to neighbouring residues of similar size, which is not uncommon for an acidic residue in single-particle cryo-EM at this resolution. Other subtle differences that may contribute to potential alterations in $G\alpha_s$ recruitment of the A316T mutant include a rotation of R176^{2,46} closer toward the $\alpha 5$ helix

of $G\alpha_s$ by 2 Å and another independent rotation of H387 slightly from its position compared to the wild-type GLP-1R structure (Figure 3.4G).

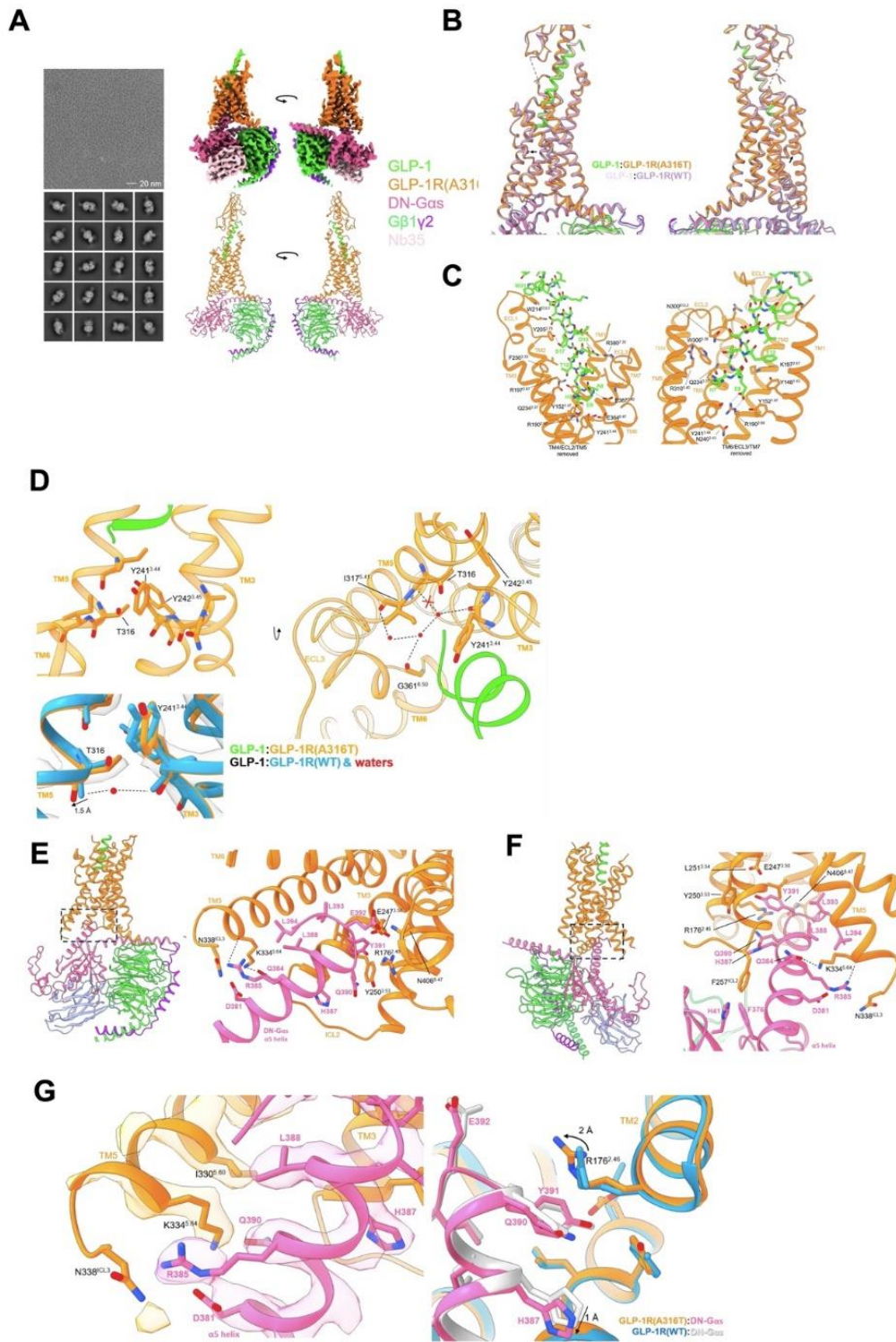


Figure 3.4 Characterisation of the Cryo-EM structure of GLP-1: GLP-1R (A316T: DN- $G\alpha_s$:Nb35. A) Representative micrograph from 200 kV cryo-EM imaging of the complex (top), with representative 2D class averages of particles used in the consensus refinements (bottom). (Right) non-uniform refined density map used as the primary source for modelling (top), with atomic model built into the density map, including backbone trace of ECD (bottom). **B)** Transmembrane domains, rendered as cartoons, of GLP-1R (A316T) (orange) and GLP-1R (WT) lavender overlaid. Black arrow indicates 316 position. **C)** Binding arrangement of residues 7-31 of GLP-1 (green) with GLP-1R (A316T) (orange) facing inwards from TM5 or from TM6. Dashed lines are hydrogen bonds predicted by ChimeraX 1.5. **D) (top left)** Positioning of T316 and Y241^{3,44} below the binding site for GLP-1 (green). **(bottom left)** Comparison between local arrangement of arrangement of residues T/A3165.40 and Y2413.44 in the A316T variant (orange) and wild-type GLP-1R (azure blue; PDB 6X18). **(right)** Intracellular-facing view of the receptor core, showing the positioning of the TM3-TM5-TM6 structural water network (red) from PDB 6X18. **E)** Key interactions between the GLP-1R (A316T) intracellular cavity (orange) and the $\alpha 5$ helix of DN- $G\alpha_s$ (pink) with ‘forward’ view indicated by top left cartoon. Hydrogen bonds, determined by ChimeraX 1.5, are dashed lines. **F)** Alternate view of GLP-1R (A316T) – $\alpha 5$ helix interactions as indicated by top right cartoon. **C.** Cryo-EM density map used to model interactions depicted as coloured transparent silhouettes. There is no density for N338^{ECL3}, while other nearby sidechains are well-resolved. **G)** Comparison between GLP-1R (A316T) – $\alpha 5$ helix (orange and pink) and GLP-1R wildtype – $\alpha 5$ helix (blue and white) interactions.

To explore these observations further, 3D variability analysis (3DVA) in cryoSPARC 3.2 and a 3-principal component analysis (PCA) model, was employed by our collaborators on the particles used in the consensus refinement. 3DVA, is a powerful tool for examining conformational heterogeneity in cryo-EM datasets. Previous analyses of the GLP-1R (WT): GLP-1 cryo-EM structures with peptide ligands have demonstrated three modes of motion: a coordinated twisting motion of receptor TMD; a large oscillation of the ECD and peptide C-terminus; and a rocking/twisting of the receptor and G protein. Unexpectedly, a striking increase in GLP-1R (A316T) ECL3 variability was observed compared to the GLP-1R (WT), and this was accompanied by more extreme movements in TM6 and the N-terminal half of the peptide (Figure 3.5A). Despite this divergent motion being particularly evident in principal component 3, it was observed in all three principal components and was preserved at low contour. Lastly, comparison of the 3DVA of the GLP-1R: GLP-1(WT) with that of the A316T

revealed that TM6-ECL3-TM7 underwent a larger motion down and away from the receptor core (Figure 3.5A, B).

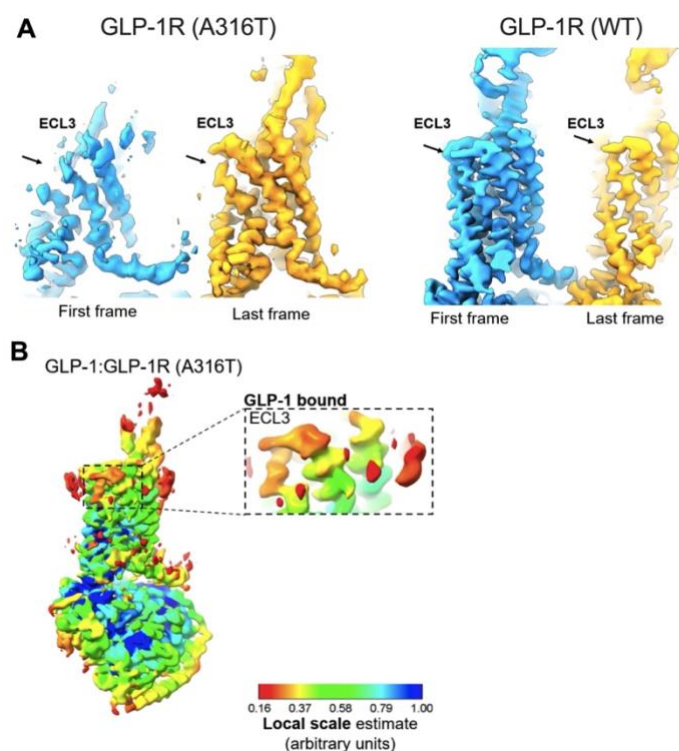


Figure 3.5 Structural Dynamics of GLP-1R (A316T) in complex with GLP-1. **A)** Closeups of the first and last frames from principal component 3 after 3DVA of GLP-1R (A316T) and GLP-1R (WT) with density clipped for clarity. **B)** Local scale estimation of GLP-1 bound GLP-1R (A316T).

3.2.4 Cryo-EM determination of the exendin-D3: A316T GLP-1R: $G\alpha_s$ complex

Our collaborators also resolved the cryo-EM structure of GLP-1R(A316T): exendin-D3: DN- $G\alpha_s$ at a 3.3-3.4 Å resolution to probe the conformation of this receptor variant with an agonist with a slower dissociation rate compared to GLP-1 (Figure 3.6A). The majority of TMDs, ECL2, ECL3 of the A316T receptor and the DN- $G\alpha_s$ were visible in the structure, but not the extended C-terminus of exendin-D3 due to its mobility. Similar to the GLP-1R(A316T): GLP-

1 complex structure, the positioning of the GLP-1R(A316T): Exendin-D3 TMDs, the orientation of the G protein, and the pattern of binding interactions of the receptor with the TMD is very similar to that of the GLP-1R (WT): exendin-4: $G\alpha_s$ complexed structure (Figure 3.6B). Moreover, the interface between the $\alpha 5$ helix of DN- $G\alpha_s$ and the intracellular face of exendin-D3-bound GLP-1R (A316T) is similar to that of the GLP-1(7-36)NH₂-bound structure (Figure 3.6D). However, unlike the GLP-1 bound structure, D381 of $G\alpha_s$ is in a more inward-facing conformation that would allow hydrogen bonding with K334^{5.64} as well as with Q384, and it exhibits a thickening of the density around T316 and central TM5, indicating greater flexibility and mobility in this region (Figure 3.6E). If the T316 is modelled closer to the central TM3 network, it can form hydrogen bonds with the carbonyl backbone of Y241^{3.44} bypassing the TM3-5-6 network (Figure 3.6E).

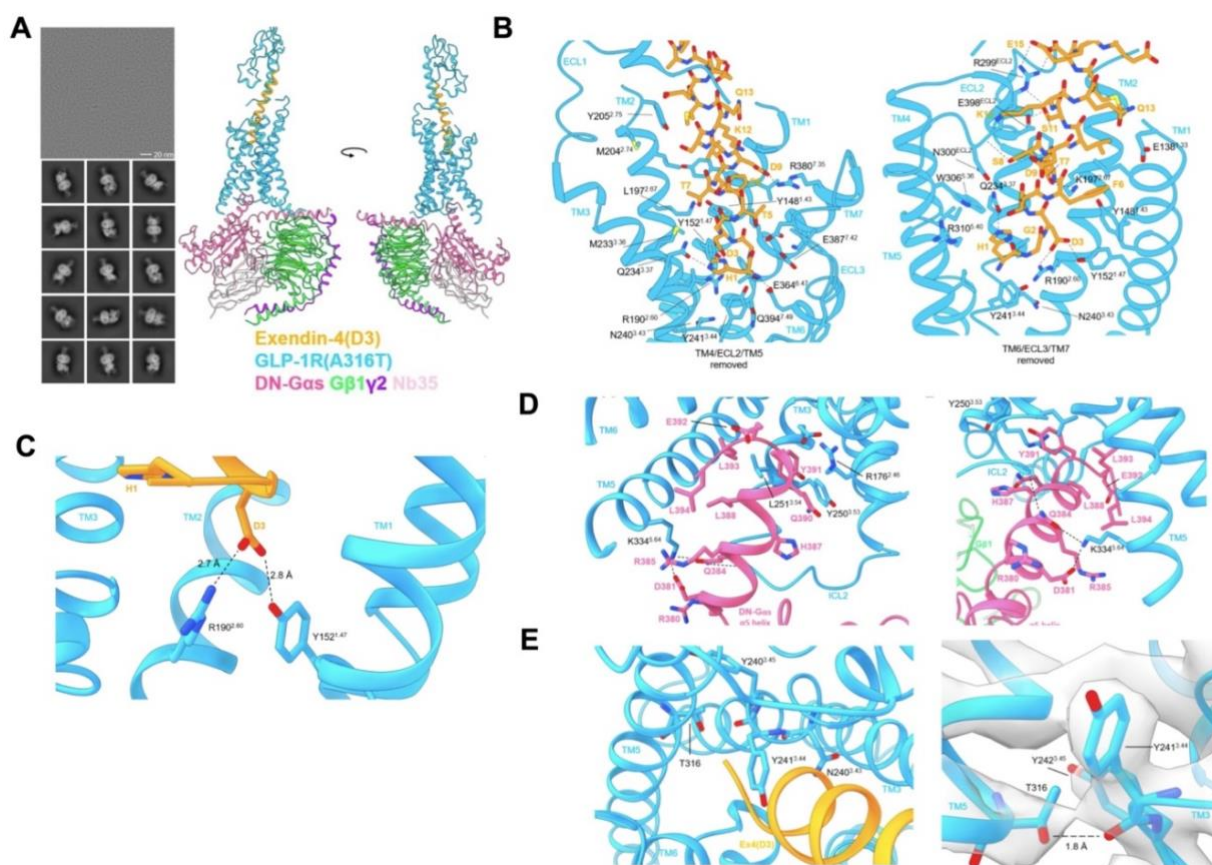


Figure 3.6 Characterisation of the Cryo-EM structure of Exendin-D3:GLP-1R (A316T):DN-Gas:Nb35. **A)** Representative micrograph of the vitrified exendin-4(D3):GLP-1R(A316T):DN-Gas:nb35 complex collected at 200 kV and (bottom) representative 2D class averages from the final particle set. **B)** Secondary structure cartoon of the atomic model for the complex. **C)** Binding arrangement of residues 7-31 of exendin-4(D3) (orange) with GLP-1R (A316T) (blue), facing inwards from TM5 (left) and facing inwards from TM6 (right). **D) Left:** Key interactions between the exendin-4(D3)-bound GLP-1R (A316T) intracellular cavity (orange) and the $\alpha 5$ helix of DN-Gas (pink) with ‘forward’ view indicated by top left cartoon. **Right:** Alternate view of GLP-1R (A316T) – $\alpha 5$ helix interaction. Hydrogen bonds, determined by ChimeraX 1.5, are dashed lines. **E) Left:** Positioning of T316, Y241^{3,44} and neighbouring residues below the binding site for exendin-4(D3) (orange). **Right:** Local arrangement of residues T316 and Y241^{3,44} with density map at medium contour (transparent grey).

As before, 3DVA using cryoSPARC was performed to compare the GLP-1R(A316T): GLP1 and the Exendin-D3, whereby our collaborators observed that the exendin-D3 bound structure appears to be less dynamic at the TM6-ECL3-TM7 region, yet the ECL3 exhibits increased stability (Figure 3.7A). To explore ECL3 stability quantitatively, OccuPy was used to estimate the local contrast degradation of the final reconstructions for each peptide. This tool allows the relative signal-to noise ratio across the density map to be calculated, allowing for regions of high and low contrast degradation to be determined. Variation in local scale across the GLP-1R (A316T) density directly represents differences in flexibility of these regions. Following this, estimates show a reduced scale in GLP-1- *versus* exendin-D3 bound structures, in the ECL3, top of TM7, and ECL1 regions (Figure 3.7B). It seems likely that these differences in local scale are driven by increased flexibility of ECL3, ECL1, and the peptide itself in the GLP-1 bound structure. The reduced dynamics in the ECL3 region is consistent with the crucial role this has in exendin-4 agonism. If this region is truly more dynamic in the Exendin-D3 bound variant structure, then it is possible that peptide cycling (disengagement and re-engagement with the receptor) could be faster, resulting in increased signalling events per unit time compared to the wild-type.

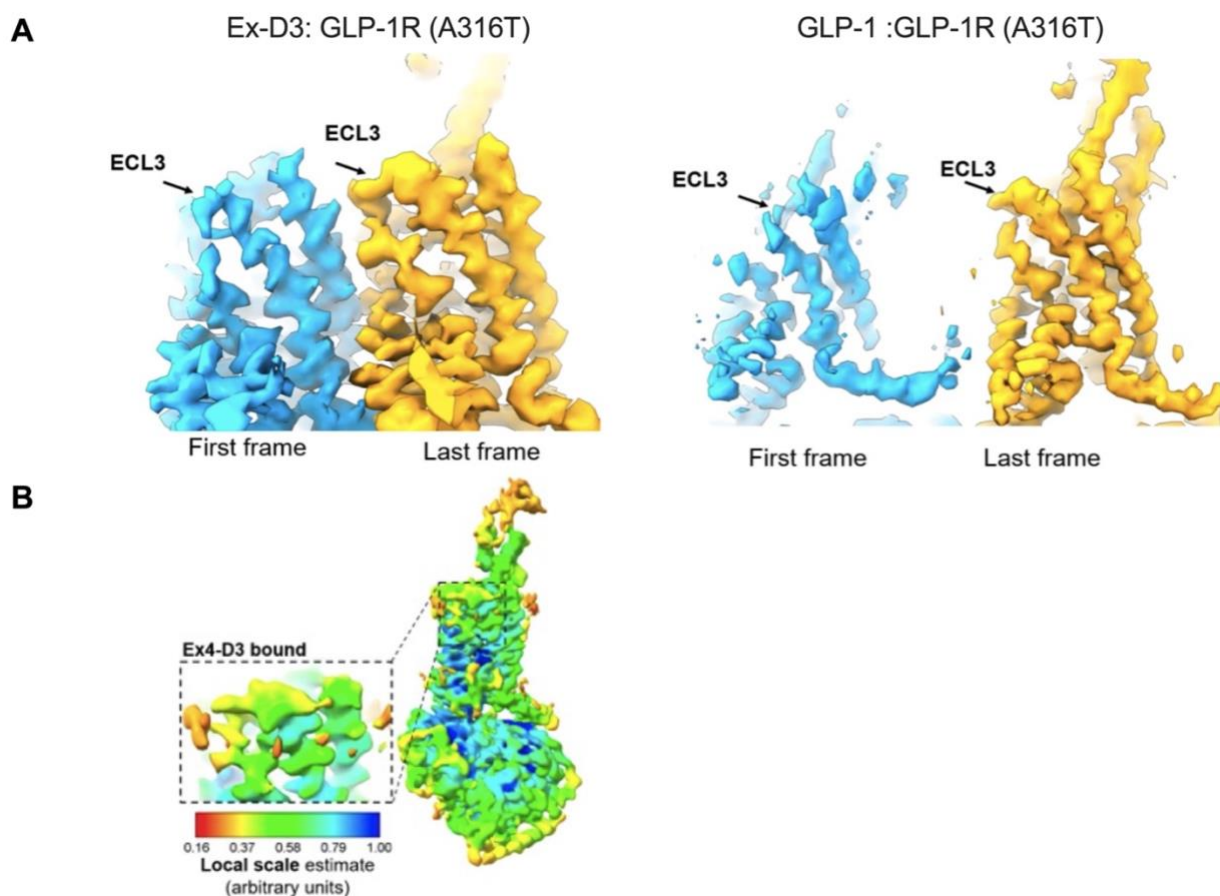


Figure 3.7 Structural Dynamics of GLP-1R (A316T) in complex with Exendin-D3. **A)** Comparison of the 3DVA Principal component 2 of exendin-D3-bound GLP-1R (A316T) (Left) to GLP-1-bound GLP-1R(A316T) (Right). **B)** Local scale estimation of exendin-D3 (Ex-4-D3)-bound GLP-1R (A316T).

3.2.5 MD simulations using cryo-EM A316T GLP-1R structures

In light of the new observations in the cryo-EM structures of GLP-1: GLP-1R(A316T):DN- $G\alpha_s$ complexes, our collaborators at Coventry sought to retrieve atomistic insights about the effect of the A316T mutation on the structure and dynamics of the GLP-1R using these structures. Additional MD simulations were conducted on the GLP-1R A316T and WT structures in three different configurations: i) the isolated TMD in the absence of any agonist (using the PDB 6X18); ii) in complex with the endogenous agonist GLP-1 (i.e. using the new

GLP-1:GLP-1R(A316T): DN- $G\alpha_s$ presented in the previous section and the PDB 6X18); and
iii) in complex with exendin-F1 (i.e. the WT cryo-EM unpublished structure from Denise Wootten at Monash University, and a modelled A316T structure based on the former).

From these MD simulations our collaborators observed that the A316T mutation caused a significant reshuffling of hydrogen bonds within the GLP-1R (Table 3.2). Specifically, the T316^{5.46} side chain in the mutant formed a hydrogen bond with Y242^{3.45}, which replaced the bond between Y242^{3.45} and the P312^{5.42} backbone seen in the GLP-1R WT structure. This shift was particularly noticeable in the isolated TMD configuration in the absence of any agonist, where the persistence of the Y242^{3.45}-P312^{5.42} hydrogen bond dropped dramatically from 95.2% in the WT to just 2.2% in the A316T mutant. Yet, similar less pronounced reductions in persistency were also observed in the GLP-1 or exendin-F1 and $G\alpha_s$ complexes, with the hydrogen bond persistency dropping from 93.3% to 46.1% in the former and from 87.4% to 7.9% in the latter. The higher persistence of this hydrogen bond formed as a result substitution of A316^{5.46} to the hydrophilic T316^{5.46} instead of the P312^{5.42} in the GLP-1-bound complex compared to the exendin-F1-bound complex, suggests that this occurs in a ligand dependent manner.

Table 3.2 Hydrogen bonds reshuffling in the proximity of position 316. WT GLP-1R and A316T were simulated in three different configurations: the isolated TMD in the absence of any agonist, in complex only with the endogenous agonists GLP-1, and in complex with exendin-F1 (Ex-F1) and $G\alpha_s$.

Residue 1	Residue 2	WT GLP-1R TMD (% MD frames)	A316T GLP-1R TMD (% MD frames)	WT GLP-1R:GLP-1 (% MD frames)	A316T:GLP-1 (% MD frames)	WT GLP-1R:Ex-F1-F1:Gs (% MD frames)	A316T:Ex-F1:Gs (% MD frames)
Y242 ^{3.45} (sc)	A/T316 ^{5.46} (bb/sc)	0.0	85.3	0.0	41.8	0.0	37.7
Y242 ^{3.45} (sc)	P312 ^{5.42} (bb)	95.2	2.2	93.3	46.1	87.4	7.9
A/T316 ^{5.46} (bb/sc)	P312 ^{5.42} (bb)	0.0	97.2	0.0	49.6	0.0	86.9
A/T316 ^{5.46} (bb/sc)	I313 ^{5.43} (bb)	0.0	1.6	0.0	47.2	0.0	8.3

Interestingly, these MD simulations also led to the discovery that in the GLP-1R (A316T) mutant, the T316^{5.46} residue exhibited two distinct rotameric states. In the agonist independent TMD, State 1 was predominant, resulting in a high persistence of the Y242^{3.45}-T316^{5.46} hydrogen bond (85%) (Figure 3.8A, Table 3.2). Conversely, in the presence of the endogenous agonist GLP-1 and $G\alpha_s$, the side chain of the T316^{5.46} flipped between State 1 and another conformation State 2, leading to formation of alternative hydrogen bonds with the backbone of I313^{5.43} and a water bridge with Y241^{3.44} backbone (State 2 in Figure 3.8B, Table 3.2). This hydrogen bond reshuffling aligns with previous MD simulations of oxyntomodulin and $G\alpha_s$ -complexed GLP-1R performed in the Lagou *et al.* (2023) study, whereby the Y242^{3.45}-P312^{5.42} hydrogen bond in the WT (occupancy 88.9%) was replaced by the Y242^{3.45}-T316^{5.46} hydrogen bond in the A316T mutant (occupancy 62.3%). This change was due to consistent adoption of the rotameric State 1 throughout the simulations, indicating an allosteric effect of the agonist

on T316^{5.46} dynamics and, consequently, the Y242^{3.45}-T316^{5.46} hydrogen bond. Cryo-EM maps described in the previous section further confirm a double rotameric state for T316^{5.46}, accommodating both “upward” and “downward” side-chain orientations.

Table 3.3 Water-mediated interactions involving position 316. WT GLP-1R and A316T were simulated in three different configurations: the isolated TMD in the absence of any agonist, in complex only with the endogenous agonists GLP-1, and in complex with exendin-F1 and G α_s .

Residue 1	Residue 2	WT GLP-1R TMD (% MD frames)	A316T GLP-1R TMD (% MD frames)	WT GLP- 1R:GLP -1 (% MD frames)	A316T GLP- 1R:GLP -1 (% MD frames)	WT GLP- 1R:Ex-F1:Gs (% MD frames)	A316T:GLP- 1R: Ex- F1:Gs (% MD frames)
A/T316 ^{5.46}	Y241 ^{3.44}	12.2	2.0	3.1	29.9	11.2	0.8
A/T316 ^{5.46}	N320 ^{5.50}	3.9	1.1	0.6	5.2	3.4	1.1

State 2, although less stable than State 1, plays a significant role in GLP-1R dynamics specifically in the presence of distinct agonists. In the A316T GLP-1R, the T316^{5.46} side chain in State 2 affects the formation of hydrogen bonds involving N320^{5.50}. The N320^{5.50} residue plays a crucial role in the structural integrity and dynamics of the GLP-1R. In the GLP-1R (WT), N320^{5.50} consistently forms a stable hydrogen bond with the backbone of L360^{6.49} (TM6 kink) across all configurations, with occupancies of 65.8%, 79.6%, and 65.4% in the isolated TMD, GLP-1 complex, and exendin-F1 and G α_s complex, respectively (Table 3.4). This stable interaction is critical for maintaining the receptor's structural stability. In contrast, in the GLP-1R A316T, this hydrogen bond was notably weakened, with a drop in occupancy to 30.6% in the isolated TMD and 35.6% in complex with GLP-1, indicating a significant loss of stability (Figure 3.8A, Table 3.3). Yet, in the GLP-1R (A316T): exendin-F1: G α_s complex, this reduction was less pronounced (54.8%).

Table 3.4 Water-mediated interactions involving N320^{5.50}. WT GLP-1R and A316T were simulated in three different configurations: the isolated TMD in the absence of any agonist, in complex only with the endogenous agonists GLP-1, and in complex with exendin-F1 and G α_s .

Residue 1	Residue 2	WT GLP-1R TMD (% MD frames)	A316T GLP-1R TMD (% MD frames)	WT GLP- 1R:GLP -1 (% MD frames)	A316T GLP- 1R:GLP -1 (% MD frames)	WT GLP- 1R:Ex- F1:Gs (% MD frames)	A316T:GLP- 1R: Ex-F1:Gs (% MD frames)
N320 ^{5.50} (sc)	L360 ^{6.49} (bb)	68.5	30.6	79.6	35.6	65.4	54.8
N320 ^{5.50} (wb)	L360 ^{6.49} (wb)	19.5	49.3	12.2	26.8	24.2	31.7
N320 ^{5.50} (wb)	G361 ^{6.50} (wb)	5.3	19.5	5.9	10.3	10.9	9.8

This weaker water bridge mediated direct interaction with N320^{5.50} is compensated by stronger water mediated interactions in the A316T variant, which help maintain the receptor's structural integrity despite the reduced strength of HB. These interactions involved N320^{5.50} and L360^{6.49}, as well as N320^{5.50} and G361^{6.50}, particularly in the presence of GLP-1 and in the isolated TMD. For instance, in the GLP-1 complex, water-mediated interactions between N320^{5.50} and L360^{6.49} increased from 12.2% in the WT to 26.8% in the A316T mutant. Similarly, in the isolated TMD, these interactions rose from 19.5% to 49.3%. This increase in water-mediated interactions was due to deeper water molecule permeation into the polar core of the receptor in the A316T mutant, compared to the GLP-1 bound GLP-1R (WT) (Figure 3.8C-H). In fact, a significant increase in water molecule residence time (up to 15%) was observed in the GLP-1R (A316T) when complexed with GLP-1 or Ex-F1 and G α_s (Table 3.5). This increase was not seen in the isolated TMD structure, where water was exchanged with

bulk solvent rather than being stabilized as structural water molecules, as in the agonist-complexed A316T configurations.

Table 3.5 Water molecules maximum occupancy nearby positions 316, 320,360 and 361. The occupancy of the water molecule with the highest residence time is reported as % of the total MD frames.

Residue 1	WT GLP-1R TMD (% MD frames)	A316T GLP-1R TMD (% MD frames)	WT GLP-1R:GLP-1 (% MD frames)	A316T GLP-1R:GLP-1 (% MD frames)	WT GLP-1R:Ex-F1:Gs (% MD frames)	A316T:GLP-1R: Ex-F1:Gs (% MD frames)
A/T316^{5.46}	1.1	1.8	0.8	6.8	1.5	5.9
N320^{5.50}	2.6	4.4	3.8	15.3	4.4	11.8
L360^{6.49}	2.3	3.8	2.7	11.3	3.0	7.3
G361^{6.50}	2.4	3.1	9.9	13.2	6.7	14.9

To gain deeper insights into the possible allosteric effects of the persistent Y242^{3.45}-A316T^{5.46} hydrogen bond in rotameric State 1, MD simulations of the isolated TMD of GLP-1R were further analysed. Our collaborators considered that structures other than the TMD (such as the ECD and ECL) and binders (GLP-1 and G α_s) could directly or allosterically influence the TMD conformations resulting from the A316T mutation, potentially masking subtle allosteric effects. Indeed, they found that the persistent Y242^{3.45}-A316T^{5.46} HB in the GLP-1R (A316T) mutant caused structural shifts in the TMD of the receptor, moving TM3 and TM5 away from each other, as depicted by the increase in distance between the backbone residues L245^{3.48} and V319^{5.49} (Figures 3.8B and C). This effect was consistent with cryo-EM data showing that the bulkier A316T^{5.46} side chain in A316T displaced its backbone carbonyl by approximately 1.5 Å from the interhelical space compared to WT.

Furthermore, the intracellular distance between TM3 and TM5 at the L251^{3.54}-V327^{5.57} level shortened by about 1 Å in the A316T mutant (Figure 3.8B and C), supported by a more

persistent hydrogen bond between Y252^{3.55} and R326^{5.56}. This is consistent with an established allosteric GPCR effect in which structural contractions or expansions at the extracellular or intracellular side influence receptor dynamics at the opposite side (575). In concert with the TM3-TM5 intracellular contraction, the TM6 outward opening, a hallmark of GPCR activation to accommodate $G\alpha_s$ coupling, was about 2.5 Å wider in the isolated TMD of A316T compared to WT (Figure 3.8D). However, this TM6 wider opening was not observed in the GLP-1R A316T: GLP-1 complex, suggesting that the agonist can modulate the TM3-TM5 distance by influencing the rotameric state of T316 and, consequently, the formation of the Y242^{3.45}-T316^{5.46} hydrogen bond.

In summary, these MD simulations show that the A316T polymorphism significantly alters the structural dynamics and hydrogen bonding interactions of GLP-1R, with these effects being modulated by the presence of different agonists.

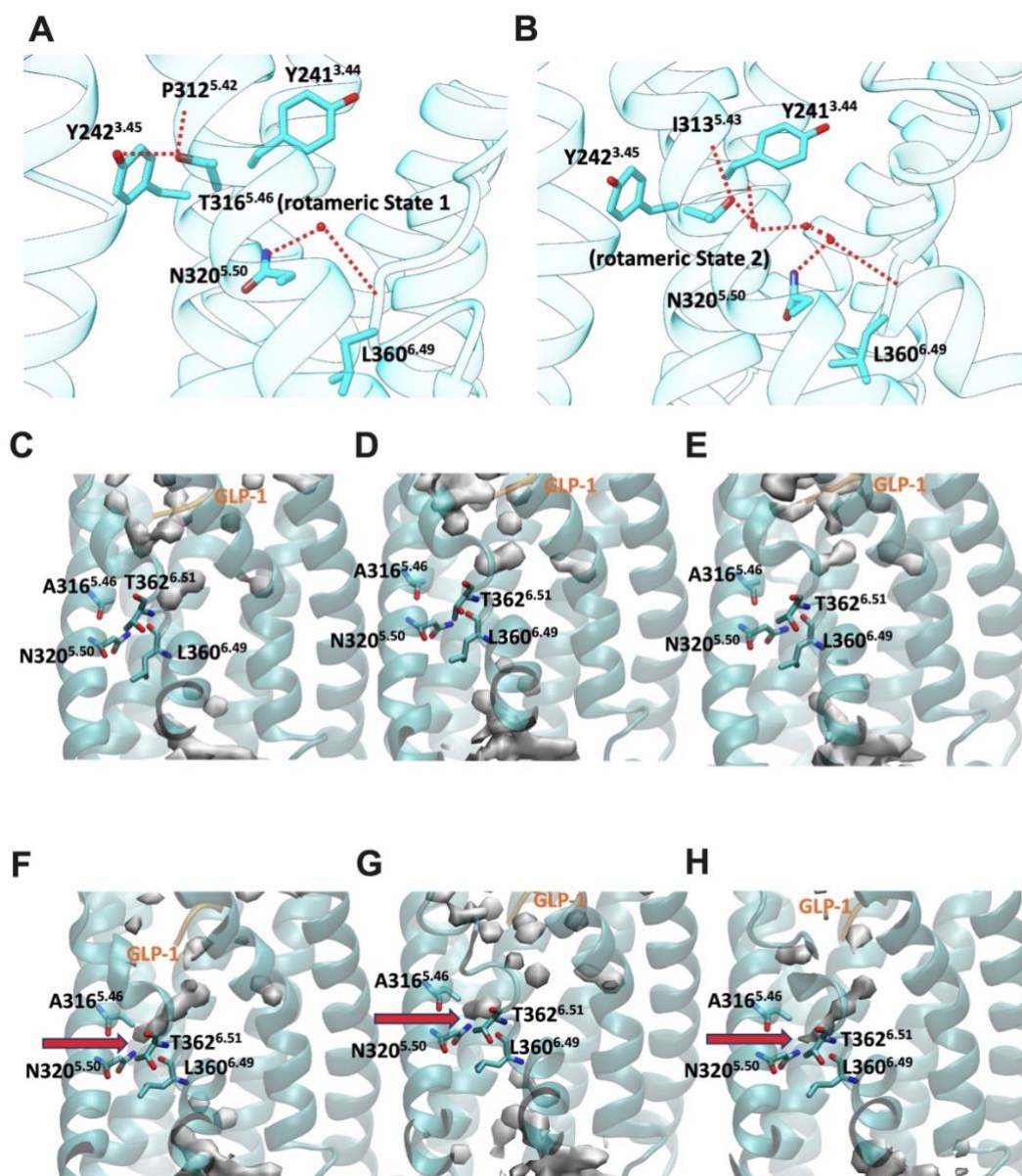


Figure 3.8 Divergent hydrogen bond and solvation patterns in WT GLP1 and A316T. A) T316^{5.46} rotameric State 1 forms a stable hydrogen bond with Y242^{3.45} B) T316^{5.46} rotameric State 2 is involved in water-mediated interactions with the polar core of the receptor. N320^{5.50} forms stable water bridges with TM6 kink (L360^{6.49} and G261^{6.50}) in A316T; C-E) volumetric map (isovalue 0.3) of water molecules within the TMD of WT GLP-1 (cyan ribbon) in complex only with the endogenous agonists GLP-1 (orange ribbon) from three independent MD simulation replicas; F-H) volumetric map (isovalue 0.3) of water molecules within the TMD of A316T (cyan ribbon) in complex only with the endogenous agonists GLP-1 (orange ribbon) from three independent MD simulation replicas. The red arrows indicate the position of stable water molecules interacting with N320^{5.50} and TM6 kink.

3.3 Discussion

In this chapter, MD simulations of ligand-free and ligand-bound as well as cryo-EM structures of the A316T variant of GLP-1R in complex with G proteins and two different peptide agonists were presented. The goal of these experiments was to use structural biology and bioinformatics to understand the molecular mechanisms underpinning the altered pharmacology of this clinically significant variant.

3.3.1 Predicting changes in receptor dynamics and receptor-residue interactions using MD simulations

In general, MD simulations of the ligand-free A316T complex suggest that the variant influences the ECD to adopt partially active conformations and dynamics. Moreover, these simulations in conjunction with inter-residue distance measurements suggest that the A316T mutation appears to influence the receptor to adopt an intermediate-active-like conformation via decreasing the distance between TM6 and TM7, and making the ECD less flexible and more open, allowing peptide binding and interaction. This also suggests that a closer distance between TM6 and TM7 correlates with less flexibility and increased opening of the ECD, signifying a partially active state in the absence of ligand binding. Previous studies show that the movement of the ligand into the receptor binding pocket shifts TM7 towards TM6, contributing to TM6 rearrangement and receptor activation (561). Interaction between charged residues in the N-terminal of the GLP-1R A316T ECD and TM6 likely confers TM6 flexibility and outward movement whilst maintaining the stability of the receptor. Thus, we can conclude

that the inactive peptide-free A316T state likely adopts a partially active state by altering hydrogen bonding networks within TM3 and TM5 domains, increasing TM6-TM7 distance proximity and thus TM6 outward movement, resulting in reduced flexibility and exaggerated opening of the ECD domain whilst maintaining stability of the variant receptor.

Next, our collaborators at Coventry have addressed the pharmacological differences between the WT and A316T agonist bound structures, rationalizing them from a molecular point of view with MD simulations with the GLP-1R agonists GLP-1, exendin-4, and its analogues exendin-F1 and exendin-D3, along with the $G\alpha_s$ protein. The RMSF analysis revealed distinct flexibility patterns depending on the ligand bound. When the GLP-1R (A316T) is bound to GLP-1, it exhibits reduced flexibility in regions such as the ECD, ECL1, and ICL3, but increased flexibility in the TM6 kink and transmembrane domains (TM) 2,3,4 and 6. Contrastingly, with the exogenous agonist exendin-4 bound, there were no notable differences in flexibility at the mutation site, but increased dynamics in the ECD's stalk region, ECL1 and slight changes in TM3-5. Nonetheless, the A316T mutation led to increased flexibility in the whole receptor when bound to the $G\alpha_s$ biased exendin-4 analogue exendin-F1, with marked modifications in receptor dynamics seen in the ECD, TM1, TM3-5, and various loops (ECL1,2,3/TM7) regions.

Finally, binding of this mutant receptor to the other β -arrestin 2 biased exendin-4 analogue, exendin-D3, elicited alterations in receptor dynamics, with changes in the ECD stalk, ECL1-2, TM5, the region TM6/ICL3/TM7, and the CTER helix. Agonist-induced conformational changes in the A316T GLP-1R were not limited to receptor dynamics but also included intra-receptor residue interactions. The A316T GLP-1R exhibited persistent interactions between T316^{5,46} with P312^{5,42} and Y242^{3,45}, mirroring the ligand-free receptor, but varied with binding

to different ligands. When bound to exendin-4 or its analogues, these interactions were present consistently, but upon GLP-1 binding, the interaction was only present half the time, suggesting more dynamic behaviour with the endogenous ligand. Instead, the GLP-1-bound A316T GLP-1R structure showed stronger interactions with the backbone of I313^{5,43}, indicating a distinct stabilisation mechanism.

Notably, differences in hydrogen bond interactions involving N320, between the ligand bound GLP-1R A316T and WT, as well as in GLP-1R A316T bound to different ligands suggest differences in TM6 kink stabilisation and thus receptor activation. Although the A316T receptor exhibited strong interactions between N320 and the backbone of L360^{6,49} and L244^{3,47} when bound to exendin-4 analogues, similar to the WT GLP-1R bound to exendin-4. The WT receptor exhibited stronger interactions with G361^{6,50}, L244^{3,47}, and L245^{3,48} in complex with exendin-4 analogues exendin-F1 and -D3. In conclusion, persistent interactions of T316^{5,46} with P312^{5,42} and Y242^{3,45} and specific changes in N320 interactions depending on the ligand bound suggest a complex mechanism of receptor activation and signalling modulation. These findings identify these residues as important hubs by which the mutant receptor modulates its function and signalling. Thus, future studies should employ site directed mutagenesis to generate alanine substitutions at these sites, specifically N320^{5,50} as it is involved in a crucial hydrogen bonding network association with the allosteric effects of G protein binding (576).

3.3.2 Cryo-EM determination of GLP-1 and exendin-D3-bound GLP-1R (A316T) complexes

The rationale behind studies performed in this chapter is to elucidate how the A316T mutation affects the structure of the GLP-1R when bound to the endogenous GLP-1 as well as the biased exendin-D3 agonist.

Resolving the GLP-1R (A316T): GLP1:DN-G α_s :Nb35 cryo-EM structure, suggested that while being largely similar to the wild-type receptor, the variant receptor exhibits changes in key water-mediated interactions between TM5 and its neighbouring helices, which could alter transmembrane domain dynamics. Additionally, subtle changes to the variant receptor intracellular face and $\alpha 5$ helix of DN-G α_s interactions were found, which might provide insights into the pharmacological profile of the variant.

The A316T substitution introduced significant changes in crucial water-mediated interactions between transmembrane domains, specifically impacting the hydrogen bonds involved in receptor activation and signalling. Moreover, the presence of the T316 residue displaced key hydrogen bond interactions, reducing interhelical packing and stiffness, which could alter the receptor dynamic responses to ligand binding, confirming previous MD simulations of changes in the mutant receptor dynamic responses to this peptide (43). Novel structural waters bridging essential residues were also identified, suggesting potential alterations in G α_s protein recruitment and downstream signalling pathways. Reassuringly, findings from the static cryo-EM structure align with the MD simulations, revealing increased flexibility in the TM6 region and other transmembrane domains, particularly when the GLP-1 is bound. The MD

simulations also highlighted that the A316T mutation leads to increased dynamics in the ECD, ECL1 and ECL3, which can affect the overall flexibility of the receptor. Thus, flexibility in these regions could be acting in synergy to enhance receptor signalling and function, explaining previous findings showing improved mini-G_s coupling in response to GLP-1 in HEK293 cells (518).

3.3.3 MD simulations utilising cryo-EM resolved GLP-1 bound GLP-1R (A316T)

Taking into consideration the novel findings elucidated from the static cryo-EM GLP-1:GLP-1R(A316T):DN-Gα_s structure, and especially the water-mediated interactions between transmembrane domains which are potentially impacting mutant receptor function and signalling, our collaborators obtained additional insights into the structural dynamics of the A316T by conducting more MD simulations on the isolated TMD of the A316T GLP-1R in complex with GLP-1 (using the cryo-EM determined structure) and exendin-F1, modelled using the exendin-F1:GLP-1R (WT):Gα_s as a starting point. These stimulations revealed significant reshuffling of hydrogen bonds within the mutant receptor independent of agonist binding. The T316^{5.46} side chain in the mutant formed a hydrogen bond with Y242^{3.45}, replacing the bond between Y242^{3.45} and the P312^{5.42} backbone seen in the wild-type receptor, aligning with the previous MD simulations done on the apostate form of this mutant receptor. In fact, this shift was the most pronounced in the agonist-free state, as indicated by the marked reduction in hydrogen bond persistencies observed in the A316T mutant. Similar but less pronounced reductions were observed in the GLP-1 and exendin-F1 complexes, indicating that the T316^{5.46} substitution causes Y242^{3.45} to engage with the hydrophilic T316^{5.46} instead of the

P312^{5.42} backbone in a ligand-dependent manner, building upon similar findings in the previous MD simulations.

Importantly, our collaborators identified two distinct rotameric states that the T316^{5.46} residue could adopt in the mutant receptor, influencing hydrogen bonding patterns differently depending on if a ligand is bound or not. In the agonist-free TMD configuration (State 1), the side chain predominantly formed a hydrogen bond with Y242^{3.45}, while in the presence of the endogenous agonist GLP-1 (State 2), the side chain flipped between conformations, forming alternative hydrogen bonds with the I313^{5.43} backbone and a water bridge with the Y241^{3.44} backbone. This hydrogen bond reshuffling aligns with previous MD simulations, suggesting an allosteric effect of the agonist on T316^{5.46} dynamics and the Y242^{3.45}-T316^{5.46} hydrogen bond. The cryo-EM maps also confirm a double rotameric state for T316^{5.46}, accommodating both "upward" and "downward" side-chain orientations. This important finding can be leveraged in the design of GLP-1R agonists, favouring peptides that elicit state 1 over state 2. Finally, these MD simulations further emphasise the crucial role that N230^{5.50} plays in the structural integrity and dynamics of the GLP-1R. In the wild-type receptor, this residue consistently formed a stable hydrogen bond with the L360^{6.49} backbone, maintaining receptor stability. Yet, in the A316T mutant receptor, this bond was notably weakened, instead water-mediated interactions compensated for the reduced strength, helping maintain structural integrity. Moreover, the simulations showed that these increased water-mediated interactions are a result of increased water molecule residence time and deeper permeation of water molecules into the polar receptor core, further supporting the retained structural stability of the A316T receptor despite the mutation. Overall, insights into the importance of water-mediated interactions in maintaining receptor stability can also be leveraged in the

development of GLP-1R-based therapeutics, where peptides, allosteric compounds, or even small molecule compounds can be designed to promote this effect.

In summary, the A316T mutation in the GLP-1R introduces specific alterations in receptor dynamics and intra-receptor interactions, potentially affecting signalling mechanisms. High-resolution cryo-EM and computational analyses provide detailed insights into these structural changes, enhancing our understanding of the structural impact of the variant. These findings are crucial for developing targeted therapies aimed at improving glucose control and thus diabetes management by leveraging the altered structural and signalling properties of the A316T GLP-1R variant. Although these A316T associated signalling properties are inherently encoded in the receptor structure, they may still be therapeutically exploitable. For example, biased agonists or allosteric modulators could be designed to preferentially stabilize receptor conformations including residue-residue interactions and hydrogen bonds favoured by the A316T mutation. Future research should continue to explore important residues highlighted by these structural and dynamic changes to fully understand their implications for receptor function and drug design. For example, the N230^{5,50} and Y242^{3,45}, which play important roles in receptor structural integrity and TMD dynamics, respectively.

Table 3. 6 Summary of key structural and dynamic differences between WT and A316T GLP-1R under agonist-bound and apo-state conditions based on MD simulations and Cryo-EM resolved structures.

Feature	Apostate WT	Apostate A316T	Agonist bound WT	Agonist bound A316T	Expected Consequence
H-bond between T316^{5.46} and Y242^{3.45}	Absent	Absent	Present (up to ~85%)	Reduced	Allosteric effect that alters TMD dynamics and TM6 mobility
H-bond between P312^{5.42} and Y242^{3.45}	Reduced	Minimal	Present (~93-94%)	Weakened (~2-46%)	This bond is replaced creating an allosteric effect
Water mediated interactions with N320^{5.20}	Weakened (19.5%)	Strong (49.3%)	Weakened (12.2%)	Reduced (26.8%)	Maintain receptor stability and structural integrity
ECD flexibility	High	Reduced	High	Intermediate	Less dynamic ECD may impair ligand sensing
TM6 Kink Dynamics	Moderate	Increased	Moderate	Increased	Greater TM6 motion favors activation
TM6-TM7 Proximity	Long distance	Short distance	Long distance	Short distance	Enhanced proximity promotes receptor activation
Water molecule occupancy in core	Low	High	Low	High	Stronger water bridge network stabilizes peptide interface
Rotameric States	Single conformation	Two states (State 1 and 2)	Single conformation	Two states (State 1 and 2)	Conformational plasticity allowing fine tuning
Receptor Flexibility	Stable	Increased	Stable	Increased	Higher dynamics could impact G protein coupling
ECD Openness	Less open	More open	Less open	More open	Greater openness may improve peptide accommodation

Chapter 4: Impact of the rs10305492 variant in rat and human β -cells

4.1 Introduction

Understanding the processes which regulate GLP-1R control of glucose metabolism and insulin secretion is essential for advancing our knowledge of diabetes pathophysiology and for the development of enhanced incretin-based therapeutics. The GLP-1R plays a crucial role in regulating insulin secretion, glucose homeostasis, β -cell function and survival, as well as promoting gastric emptying and satiety. Interaction and activation of the GLP-1R with various endogenous and therapeutic agonists leads to differential activation of signalling and trafficking pathways, significantly impacting GLP-1R associated glucose homeostasis processes (373, 381). The complexity of this ligand-receptor system is further increased by the presence of several SNPs that are distributed across the gene encoding the receptor in humans. Therefore, SNPs such as the rs10305492 (G>A, Ala316Thr or A316T), could significantly impact GLP-1R action, potentially leading to altered insulin release and glucose tolerance. Indeed, this missense mutation is associated with enhanced glycaemic control including lower random and fasting glucose as well as a lower risk of T2D (43-45, 518, 522). Investigating this specific mutation in-depth allows us to elucidate its effects on receptor functionality, signal transduction, and overall β -cell performance.

To date, a limited amount of literature has been published on the effects of the A316T variation on GLP-1R function, and most of those have utilised heterologous expression systems like

human embryonic kidney (HEK293) cells and Chinese hamster ovary (CHO) cells (382, 527). Whilst these cells are commonly used for heterologous expression of receptors and other proteins due to the ease of genetic manipulation, they lack the native cellular context, signalling pathways, and receptor-protein interactions of pancreatic β -cells, which may limit the physiological relevance of any findings. In addition to this, functional studies in these cell lines will only measure receptor activation via artificial reporters or secondary messengers, and do not reflect natural endpoints such as insulin secretion, which is also crucial to accurately predict the physiological effect of the mutation.

Despite β -cells being the predominant cell type within the islets of Langerhans, they comprise only 1-2% of total pancreatic cells (577, 578). Therefore, isolating a homogeneous population of β -cells has proven difficult, hampering studies of human pancreatic β -cell physiology as well as studies of diabetes pathogenesis and therapeutic development. Moreover, obtaining human islet samples for the isolation of these β -cells is difficult and the availability is unpredictable. Consequently, several immortalised rodent and human β -cell lines have been developed over the last few decades that have aided in β -cell research. Although some of these lines do not respond to glucose, including RINm5F (a rat insulinoma cell line) and HIT-T15 (a hamster insulinoma cell line), with reduced or negligible glucose responsiveness (579, 580), others such as the rodent cell lines INS-1 832/3 and MIN6 B1 (581, 582), and the human cell line EndoC- β H3 retain the capacity to secrete insulin in response to a physiological range of glucose (583, 584). These cell lines offer a balance between relevance to primary β -cell biology and the practical benefits of an immortalised cell line, making them valuable verified tools in diabetes research. In addition to this, use of these immortalised cell lines reduces the need for animal sacrifice, addressing ethical concerns associated with animal research, are

readily available, and are suitable for high-throughput screening of therapeutic compounds, signalling studies, and other large-scale assays that would be impractical with primary cells.

For my studies, INS-1 832/3 and EndoC- β H3 cells have been used to study the role of the A316T GLP-1R variant in rodent and human cells, respectively. The INS-1 832/3 cell line is a subclone of the original INS-1 cell line, derived from a rat insulinoma (a tumour of the insulin-producing β -cells of the pancreas), by Dr. Claes Wolheim and colleagues in the early 1990s. The INS-1 cell line, while useful, exhibited some variability in GSIS (585). Thus, to improve the consistency and robustness of insulin secretion, Dr. Christopher B. Newgard's group screened and identified several subclones with improved GSIS, the best-performing of which was designated INS-1 832/3 (586). This subclone maintains a more stable phenotype over many passages and exhibits enhanced GSIS, making them particularly useful for studying the physiology of insulin secretion, while they also exhibit responses to GLP-1RAs and endogenous GLP-1R expression. Human EndoC- β H3 cells were developed by a collaborative effort led by diabetes researchers at INSERM (the French National Institute of Health and Medical Research), mainly Prof. Raphael Scharfmann and his team, pioneers in the creation and characterisation of these human- β -cell lines (587). They are a modified drug-inducible conditionally immortalised version of the EndoC- β H2 cells, which are in turn the second generation of EndoC- β H1 human β -cells derived from human foetal pancreas and immortalised to extend their lifespan by use of immortalising genes large T antigen of SV40 (SV40LT) and human telomerase reverse transcriptase (hTERT) (587). Unlike EndoC- β H1 cells, proliferation can be arrested in EndoC- β H2 cells by CRE mediated excision of the immortalising genes using lentiviral vectors, and in the third generation EndoC- β H3, this

excision can be achieved via tamoxifen treatment and antibiotic selection (583, 587). Thus, these human β -cells provide a more accurate and reliable *in vitro* model for human function, closely mimicking GSIS, expressing key β -cell markers, and supporting genetic manipulation, while maintaining a stable phenotype for up to five weeks in culture (587-589).

Indeed, with all genetically manipulated derived cell lines, there are limitations which users must be aware of when using them for β -cell function studies. These include potential genetic and phenotypic changes over extended periods in culture, and this may affect functional characteristics and reproducibility across experiments. Prolonged culture can also lead to loss of functionality as a result of senescence over time, and other immortalisation artifacts can alter specific cellular pathways such as apoptosis impacting physiological relevance. For INS-1 832/3 cells, although they are well-established and characterised, they are of non-human origin and as the GLP-1R variant I am studying is of a human origin, this may cause species-specific differences and limit the direct translation of experimental results to human physiology. On the other hand, limitations associated with the use of EndoC- β H3 cells include an aberrantly high expression of disallowed genes *Slc16a1* (*Mct1*) and *Ldha*, which are important in nutrient sensing and are usually repressed in mature β -cells to prevent inappropriate insulin secretion (590, 591). In addition to this, these cells display attenuated dose-dependent responses to incretins due to lower expression of the GLP-1R receptor (591). Despite their overall maturity, EndoC- β H3 cells may retain some foetal or progenitor-like features, and insulin content remains lower compared to primary β -cells (590). Furthermore, variability in the efficiency of tamoxifen-induced de-immortalization and incomplete silencing of cell cycle genes can affect consistency between experiments.

Nevertheless, cell lines offer a vast range of research possibilities, allowing manipulation such as transfection and overexpression of the human SNAP-tagged wild-type or mutant GLP-1Rs to determine the role of these mutations in altering the trafficking and signalling of the receptor, providing consistency and reproducibility of results.

This study is built on and expands on previously published reports examining the A316T variant. Based on these previous findings and structural observations from our collaborators, the hypothesis of this work is that the A316T GLP-1R will exhibit enhanced signalling and trafficking profiles in the absence of ligands mimicking a partial agonist, such that agonist responses compared to the vehicle might be attenuated or unchanged. The aim of experiments performed in this chapter was to validate previous membrane localisation, endocytosis, and mini-G_s recruitment findings in rodent and human β -cell lines as well as evaluate other factors associated with GLP-1R function such as lipid raft recruitment, clustering, β -arrestin 2 coupling, and GSIS.

4.2 Results

4.2.1 The A316T variant reduces cell surface expression of the hGLP-1R in rodent β -cells

Cell surface expression of GPCRs is a product of highly regulated dynamic processes including biosynthetic and endocytic trafficking, thus it is an important factor regulating receptor signalling. Therefore, cell surface GLP-1R levels were compared between multiclonal INS-1 832/3 *Glp1r*^{-/-} cells transiently or stably expressing the SNAP/FLAG-

tagged-hGLP-1R wild-type (SNAP-hGLP-1R^{WT}) *versus* the A316T mutant (SNAP-hGLP-1R^{A316T}) using a membrane impermeable fluorescent SNAP-tag probe and confocal microscopy. Consistently with previous studies in CHO, HEK293T and INS-1 cells (382, 518, 527), the A316T variant exhibited reduced cell surface expression compared to the wild-type GLP-1R in both transiently and stably expressing cell lines (Figure 4.1A and B). Furthermore, evaluation of SNAP-GLP-1R^{WT} *versus* A316T expression by Western blotting, or with the cell permeable SNAP tag probe BG-OG, revealed significantly reduced total levels of the A316T variant compared to the WT receptor, suggesting markedly increased basal degradation or reduced protein synthesis (Figure 4.1C, D and E).

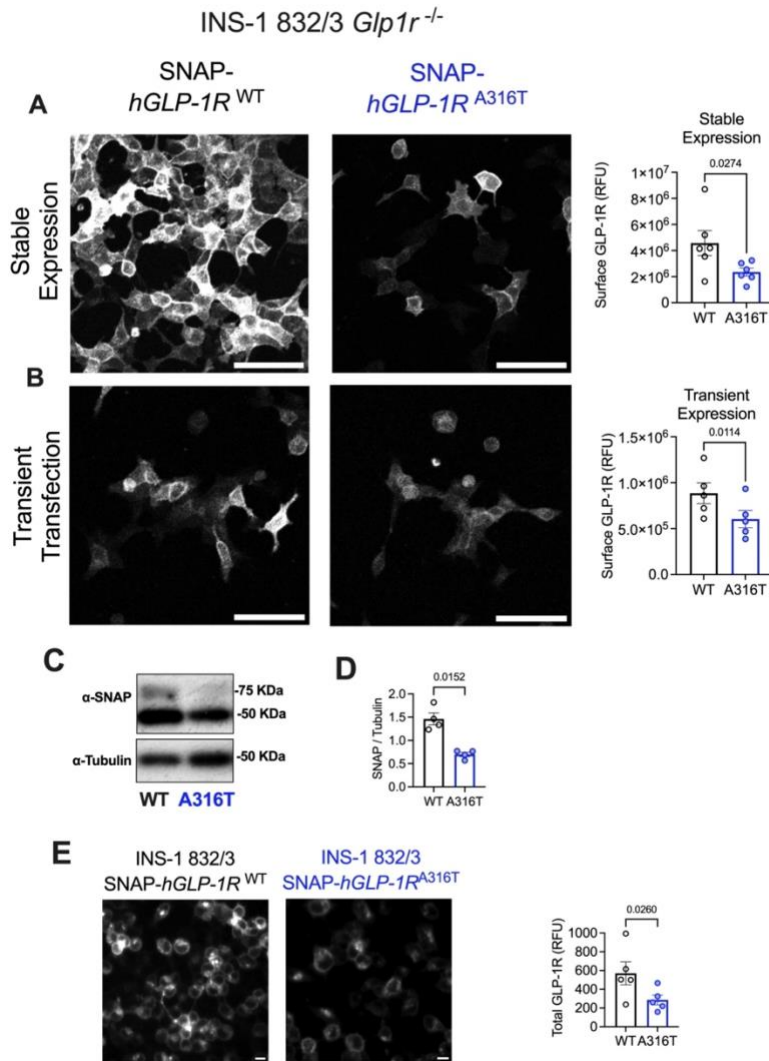


Figure 4.1 Cell Surface Expression of hGLP-1R^{WT} and the hGLP-1R^{A316T} in INS-1 832/3 *Glp1r*^{-/-} cells. **A) Comparison of hGlp1r expression between INS-1 832/3 *Glp1r*^{-/-} cells stably expressing SNAP-hGLP-1R^{WT} or hGLP-1R^{A316T}, fluorescent images from SNAP-Surface Alexa Fluor 647 labelled cells on the left and quantification of fluorescence on the right. **B)** The same as A but with cells transiently expressing the same plasmid constructs. Comparisons of A316T vs WT were made with paired-t-tests. Data are presented as means ± SEM. Scale bars = 50 μm. **C and D)** Western blot analysis of total SNAP-hGLP-1R fractions from INS-1 832/3 GLP-1R^{-/-} stably expressing SNAP-hGLP-1R^{WT} or hGLP-1R^{A316T} receptors under basal conditions. **(E)** Representative time 0 fluorescent images from BG-OG based degradation high-content microscopy assay and quantification in the same cells as above.**

4.2.2 Lysosomal inhibition rescues cell surface expression of hGLP-1R^{A316T} in rodent β -cells

To examine the underlying mechanisms responsible for the reduced cell surface expression exhibited by the A316T variant in the same rodent cell line, I treated the cells from section 4.2.1 with normal media with or without bafilomycin A1, an established vacuolar H⁺-ATPase lysosomal trafficking inhibitor (592) for 2 hours to inhibit lysosomal activity and degradation of the receptor. Expectedly, in the untreated conditions the GLP-1R A316T mutant exhibited significantly reduced cell surface expression levels. Bafilomycin A1 exposure markedly increased the cell surface expression of the A316T receptor compared to the untreated condition, rescuing cell surface expression of the mutant to levels comparable to wild-type controls (Figure 4.2A, B). This result suggests that decreased cell surface expression of the A316T variant is a result of increased basal turnover and lysosomal degradation which may be explained by the intermediate conformational state adopted by the A316T receptor in the absence of ligands conferring increased basal trafficking activity.

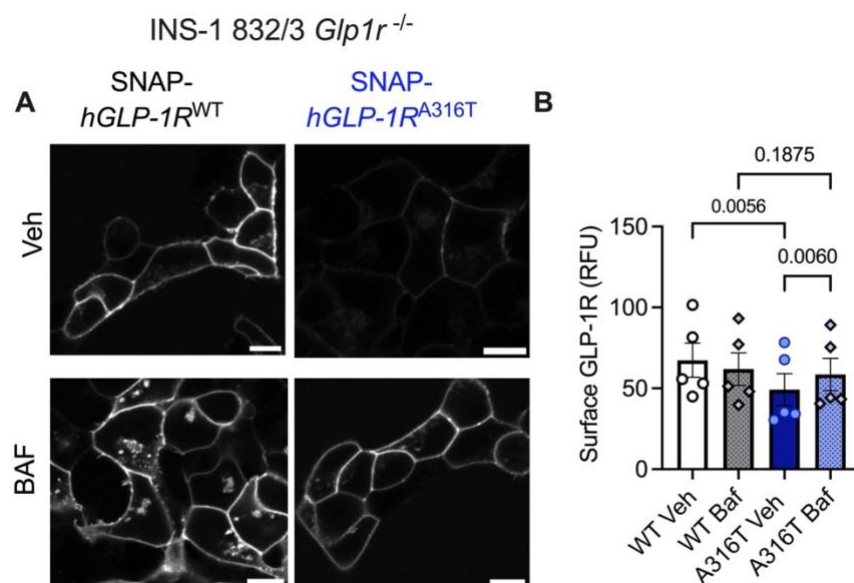


Figure 4.2 Impact of lysosomal inhibition on Surface Expression of hGLP-1R^{WT} and hGLP-1R^{A316T} mutant in INS-1 832/3 *Glp1r*^{-/-} cells. **A)** Representative fluorescent images in treated INS-1 832/3 *Glp1r*^{-/-} stably expressing SNAP-hGLP-1R^{WT} or hGLP-1R^{A316T}, pre-treated or not for 2 hours with 400uM Bafilomycin A1 (BAF), followed by labelling with SNAP-Surface Alexa Fluor 647 for 10 minutes, and **B)** corresponding fluorescence levels. Comparisons of A316T vs WT were made with one-way analysis of variance (ANOVA) with Sidak's post-hoc tests. Data are presented as means $\pm$ SEM. Scale bars= 20 μ m.

4.2.3 The A316T variant increases the ability of the receptor to induce GLP-1R conformational shifts in response to GLP-1

Previous structural studies show that the ECD of the GLP-1R WT structure adopts an extended open conformation to allow multiple interactions to form with the C-terminus of the GLP-1 along the peptide-binding groove (287). This open conformation also allows the N-terminus of the GLP-1 to access a deep pocket, rearranging the helical bundle and affecting TM movement enabling G protein recruitment efficiently, and reducing ligand concentrations needed for an endogenous or therapeutic response. Interestingly, MD studies performed by our collaborators showed that the ligand-free GLP-1R A316T structure adopts conformational

changes that resemble the GLP-1R WT: GLP1 structure, whereby distances between TM6 and 7 were closer and flexibility in the N-terminal helix of the ECD was increased, resulting in wider ECD opening.

In my assay, agonist-induced conformational changes that bring the receptor ECD closer to the plasma membrane result in an elevated TR-FRET signal between Lumi4-Tb (a luminescent terbium complex conjugated to the receptor ECD) and NR12A (a fluorescent plasma membrane marker), serving as an indicator of receptor ECD opening. In the ligand-free apstate-state there was no significant change between ECD opening in the A316T GLP-1R and the WT control (Figure 4.3A and B) , yet upon GLP-1 stimulation the A316T receptor showed increased ECD opening compared to the WT receptor across all concentrations tested, indicative of a higher degree of GLP-1R activation when bound to GLP-1 (Figure 4.3C and D). This also matches with 3DVA analysis performed by our collaborators revealing that the

GLP-1R A316T structure exhibits exaggerated TM6 outward opening movement compared to the GLP-1R WT structure.

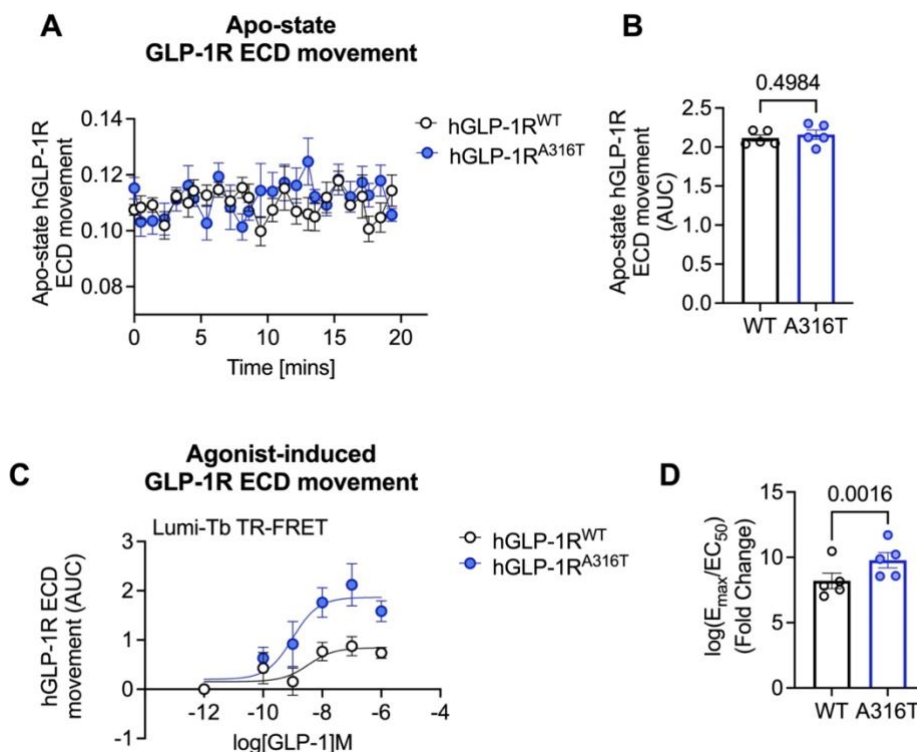


Figure 4.3 GLP-1 activation of hGLP-1R^{A316T} enhances movement of the ECD in rat pancreatic β cells. The NR12S conformational biosensor assay, with kinetic responses showing TR-FRET ratio normalized to baseline in absence of agonists in INS-1 832/3 *Glp1r*^{-/-} stably expressing SNAP-hGLP-1R^{WT} or hGLP-1R^{A316T} (A), and corresponding AUCs (B). C) Dose-response curve constructed from AUCs relating to the same assay but in response to GLP-1 at the indicated concentrations, with D) corresponding log(E_{max}/EC₅₀) efficiency calculations. AUC values are indicative of ECD opening in response to increasing GLP-1 concentrations. Comparisons of hGLP-1R^{A316T} versus hGLP-1R^{WT} were made with paired-t-tests. Data are presented as means $\pm$ SEM.

4.2.4 The A316T variant increases baseline lipid raft recruitment and clustering in rodent β -cells

Localization of membrane GPCRs in plasma membrane raft nanodomains is a known mechanism to organise these receptors into signalling micro-clusters, and this localisation can be constitutive or ligand-dependent (593, 594). Previous studies by our group revealed that stimulation with an endogenous or exogenous GLP-1R agonist, GLP-1 (7-36)NH₂ or exendin-4 respectively, resulted in increased WT GLP-1R localisation into these plasma membrane raft nanodomains (358). As the A316T variant exhibits increased basal trafficking activity and a partially active ligand-free structural state, I hypothesised that it might exhibit constitutive recruitment into these membrane nanodomains, resembling that of the ligand-activated WT receptor. To determine the influence of this variant on GLP-1R raft partitioning in pancreatic β -cells, I measured SNAP-GLP-1R levels in purified detergent-resistant and detergent-soluble fractions

from total membrane preparations of rat INS-1 832/3 *Glp1r*^{-/-} cells stably expressing human GLP-1R WT or A316T-SNAP-tagged at the extracellular N terminus (INS-1 832/3 SNAP-hGLP-1R^{WT} or ^{A316T}) both in absence and presence of GLP-1R agonists.

In vehicle conditions, the A316T variant exhibits increased recruitment to flotillin-positive DRFs compared to the WT receptor, which became significant upon correction for receptor cell surface levels (Figure 4.4A, B). Thus, this suggests that as predicted the A316T variant exhibits increased basal enrichment in membrane raft fractions compared to the WT receptor. On the other hand, whilst stimulation with 100 nM GLP-1 elicited no change in the A316T raft segregation fold responses, the opposite was true in response to the exogenous agonist exendin-4 compared the WT GLP-1R response (Figure 4.4 A, C and D). This difference in membrane nanodomain recruitment response to the GLP-1 compared to exendin-4 exhibited by the GLP-1R A316T variant is consistent with earlier findings from MD simulations showing differences in intra-receptor residue-residue interactions, hydrogen bond interactions, and receptor-ligand interactions between the GLP-1 and exendin-4 bound GLP-1R A316T complexes.

As partitioning into membrane raft nanodomains is known to regulate cell receptor clustering, and previous studies have shown a rapid increase in clustering with exendin-4 stimulation in WT GLP-1R expressing murine pancreatic β -cells (358), I next determined the degree of A316T GLP-1R clustering at the plasma membrane in the presence and absence of the endogenous agonist GLP-1. For this I first used a dual labelling approach previously described by our group which allows the detection of receptor-receptor interactions by TR-FRET. The long-lived fluorescence of the lanthanide FRET donor Lumi4-Tb, energy transfer in this assay results from both stable and transient receptor-receptor interactions, which increase when

these receptors are in close proximity. Utilising the same INS-1 832/3 SNAP-GLP-1R cells as the previous sections, I detected no change in TR-FRET levels in vehicle conditions or in following GLP-1 stimulation (Figure 4.4 E and F).

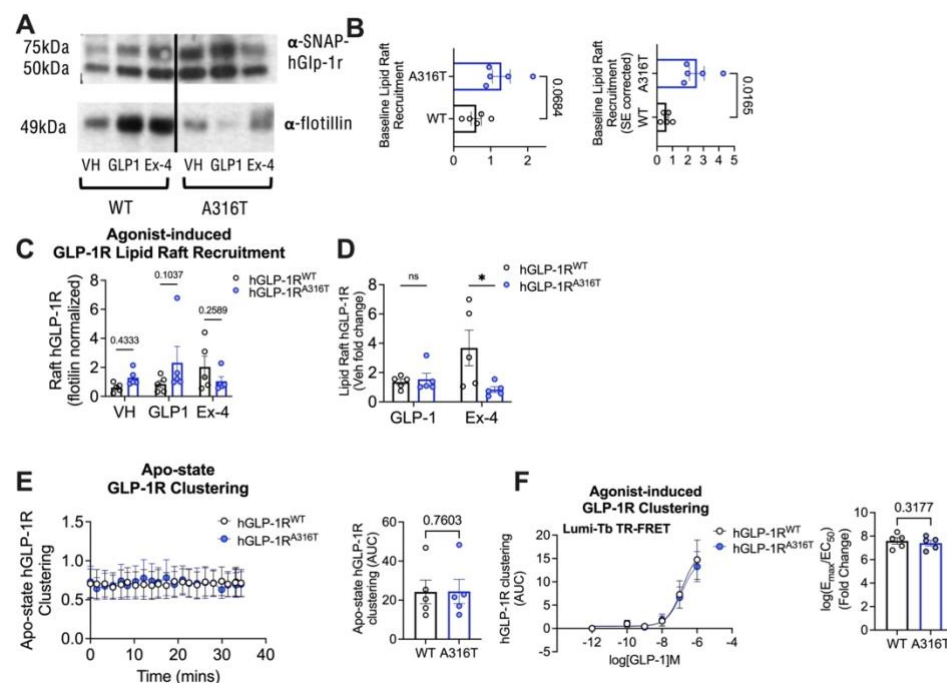


Figure 4.4 The hGLP-1R^{A316T} mutant exhibits enhanced partitioning into membrane nanodomain lipid rafts but no change in clustering independent of agonist activation. **A)** Representative blots showing SNAP-hGLP-1R distribution within DRFs (lipid nanodomains) isolated in INS-1 832/3 *Glp1r*^{-/-} stably expressing SNAP- hGLP-1R^{WT} or hGLP-1R^{A316T} receptors treated with vehicle (VH) for 2 minutes, with flotillin as a maker of membrane raft enrichment. **B)** Graphs showing quantification of SNAP-hGLP-1R levels in DRFs; individual results normalized to flotillin (raft loading control) and on the right values are further normalized to cell surface expression **C)** Results of the same assay but with cells treated with 100nM Ex-4 or GLP-1 for 5 minutes and fold changes normalized to VH **(D).** **E)** Basal clustering responses in INS-1 832/3 *Glp1r*^{-/-} stably expressing SNAP-hGLP-1R^{WT} or hGLP-1R^{A316T} cells and corresponding AUCs on the right. **(F)** Dose-response curves for GLP-1 induced clustering, with corresponding log(E_{max}/EC₅₀) efficiency calculations. Comparisons of A316T vs WT were made with paired t-tests or one-way analysis of variance (ANOVA) with Sidak's post-hoc tests. Data are presented as means ± SEM.

While the TR-FRET approach is useful for measuring close-range receptor-receptor interactions and provides quantitative information on receptor clustering, other approaches

such as Raster Image Correlation Spectroscopy (RICS) are required to provide more accurate high-resolution spatial and temporal information about GLP-1R clustering and dynamics in real time. Thus, potential changes in receptor clustering and plasma membrane diffusion were next assessed using RICS analysis of INS-1 832/3 SNAP-GLP-1R^{WT} *versus* A316T cells. The results demonstrated notably lower plasma membrane diffusion coefficients for the A316T compared to the WT GLP-1R under vehicle conditions, a pattern typically seen for the wild-type receptor only after agonist stimulation as also seen here (Figure 4.5A-C). This further corroborates my previous findings that the variant displays a phenotype consistent with constitutive activation.

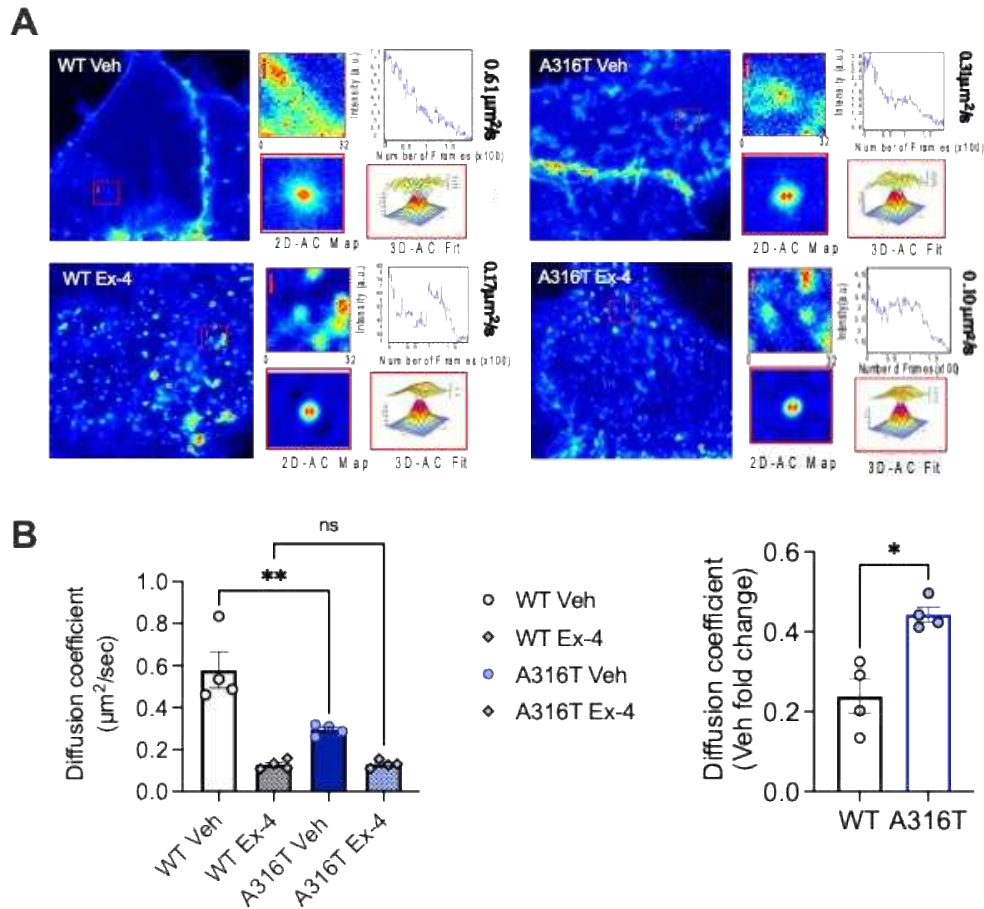


Figure 4.5 A316T versus WT GLP-1R clustering propensities in INS-1 832/3 pancreatic β cells. Representative images from RICS analysis of GLP-1R WT and A316T plasma membrane lateral diffusion in INS-1 832/3 *hGlp1r*^{-/-} SNAP-hGLP-1R^{WT} versus A316T cells labelled with SNAP-Surface Alexa Fluor 647 and subsequently stimulated with vehicle (Veh) or 100 nM exendin-4 (Ex-4). **B)** GLP-1R WT versus A316T average RICS diffusion coefficients and on the right vehicle (Veh) fold changes (**i**); $n = 4$. Figures and graphs for these experiments were generated by Dr. Affiong Iqa Oqua and Mrs. Yusman Manchanda.

4.2.5 The A316T variant exhibits agonist specific trafficking profiles in rodent β -cells

Given previous studies by our group establishing that GLP-1R WT partitioning into membrane lipid raft nanodomains and clustering is followed by internalisation of the receptor into

endosomes (358), I next investigated whether differences in trafficking characteristics of the A316T variant would reflect observations of lipid raft nanodomain localisation. To do this I used high-content microscopy analysis to assess the differences in trafficking profiles of the WT *versus* A316T mutant GLP-1R in stable INS-1 832/3 SNAP-GLP-1R^{WT} *versus* A316T cells following stimulation with a panel of GLP-1RAs including GLP-1, exendin-4, exendin-F1, and exendin-D3. For internalisation, a high content microscopy assay was used with a membrane impermeable and cleavable fluorescent SNAP-tag probe, followed by monitoring of both receptors over a period of 1 hour. Preceding agonist administration to trigger endocytosis, MesNa application cleaves residual surface fluorescence allowing the quantification of intracellular receptor from the same fields of view within each well. Results of this experiment revealed that the A316T receptor exhibited a distinct internalisation profile in response to each agonist. GLP-1 triggered increased internalisation of the A316T GLP-1R relative to the wild-type, but there was no change in response to exendin-4 and its analogues exendin-F1 or exendin-D3 (Figure 4.6C and D).

I also analysed the level of receptor recycling back to the plasma membrane after internalisation in the same cells. For this assay, I pre-treated the cells with 100nM of the GLP-1R agonists for 1 hour to trigger maximal internalisation, followed by a washout and labelling with the fluorescent GLP-1R agonist exendin-4-TMR to infer the amount of receptor reappearance at the plasma membrane. Receptors that are recycled to the cell surface will rapidly endocytose following exendin-4-TMR binding, hence the degree of recycling is based on the total (intracellular + surface) accumulation of exendin-4-TMR. Similar to the internalisation pattern, the A316T receptor showed no change in recycling compared to the

WT with exendin-4, or exendin-D3, but recycling was reduced in response to stimulation with GLP-1 and exendin-F1, yet this was only significant for GLP-1 (Figure 4.6G and H).

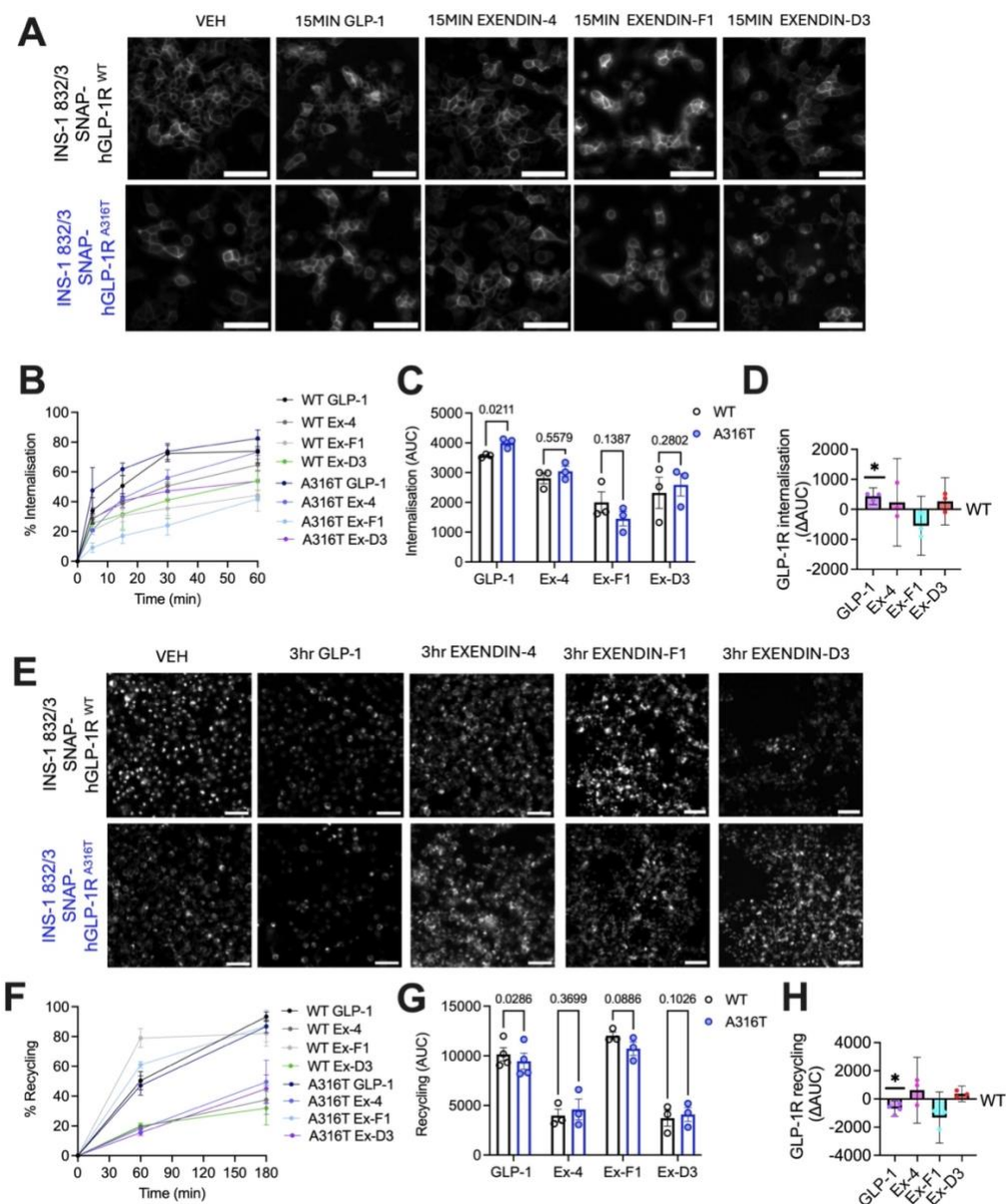


Figure 4.6 Characterisation of trafficking pathways in INS-1 832/3 SNAP-hGLP-1R ^{A316T} and wild-type control cells. **A)** Representative fluorescent microscopy images at the 15 minutes time point, upon stimulation with different agonists as indicated and corresponding fluorescent quantification (**B**). **C)** AUC calculations of agonist-induced GLP-1R internalisation in SNAP-hGLP-1R ^{WT} versus ^{A316T} cells and ΔAUCs calculated by normalising values in C to wild-type values (**D**). **E)** Recycling images and corresponding quantification of 3-hour recycling in the same cells as above and quantification of fluorescence (**F**). **G)** AUC calculations of agonist-

induced GLP-1R recycling in the same cells as above and Δ AUCs calculated by normalising values in G to wild-type values. Comparisons of A316T vs WT were made with Tukey's multiple comparisons analysis or one-way analysis of variance (ANOVA) with Sidak's post-hoc tests. Data are presented as means $\pm$ SEM for AUCs and 95% CIs for Δ AUCs. Scale bars= 50 μ m.

Finally, I assessed changes in the level of receptor degradation as a result of the A316T mutation for both the A316T and the WT receptor. For this I quantified the level of receptor degradation using a high-content microscopy approach in which remaining total cellular SNAP-tagged receptor is labelled after incubation with GLP-1RA or vehicle using the cell-permeable SNAP-tag probe BG-OG. This experiment showed that the A316T GLP-1R receptor degradation was not significantly different between the WT and A316T for any of the agonists tested (Figure 4.7A-D).

Overall, the A316T GLP-1R appeared to show distinct trafficking patterns in response to the endogenous agonist GLP-1 relative to the WT, but trafficking behaviours in response to the exendin-4 derived agonists resembled those of the WT receptor.

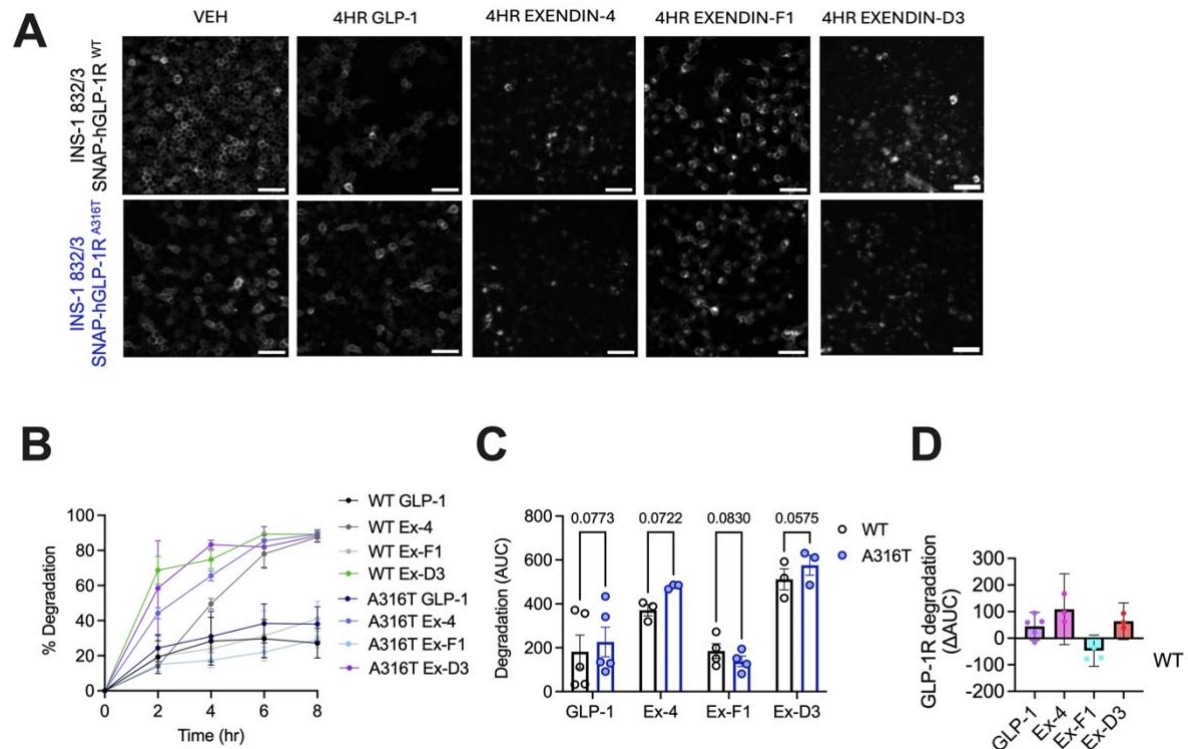


Figure 4.7 The hGLP-1R^{A316T} mutant exhibits enhanced basal lysosomal trafficking and altered agonist induced degradation profiles in rat pancreatic β cells. **A)** Representative fluorescent microscopy images at the 4-hour time point, upon stimulation with different agonists as indicated and corresponding fluorescent quantification (**B**). (**C**) AUC calculations of agonist-induced GLP-1R degradation in SNAP-hGLP-1R^{WT} versus A316T cells and Δ AUCs calculated by normalising values in C to wild-type values in (**D**). Comparisons of A316T vs WT were made with Tukey's multiple comparisons analysis. Data are presented as means $\pm$ SEM for AUCs and 95% CIs for Δ AUCs. Scale bars= 50 μ m.

4.2.6 The A316T variant exhibits increased basal G_{α_s} recruitment and changes in agonist-induced G_{α_s} bias

Agonist stimulation of the WT GLP-1R by the endogenous agonist GLP-1 or pharmacological agonists such as exendin-4 leads to the recruitment of G proteins, triggering an intracellular signalling cascade that leads to activation of downstream pathways. Previous studies by our

group showed the wild-type GLP-1R primarily couples to $G\alpha_s$, followed by $G\alpha_q$, with minimal coupling to $G\alpha_i$ in response to agonist stimulation (351). The wild-type GLP-1R also recruits β -arrestin 2 which is canonically associated with receptor desensitisation and reduced receptor efficiency. In addition to this, this pleiotropic coupling of the WT GLP-1R gives rise to bias in the recruitment of these proteins in response to differential ligand binding.

SNPs like the A316T mutation alter the structure of the receptor, potentially resulting in differential coupling to those signalling mediators and modifying GLP-1R signal transduction. Previous studies using analogous assays in HEK293T cells found that the A316T variant showed increased mini- G_s coupling responses to all ligands except exendin-4 and semaglutide (520). Similarly, others also showed that this variant exhibited a modest LoF phenotype for β -arrestin 2 and a GoF for $G\alpha_s$ pathway in HEK293T cells (529). Therefore, I predicted based on these previous findings as well as data from MD simulations, that the A316T GLP-1R might exhibit a GoF phenotype for the $G\alpha_s$ pathway independent of agonists, and agonist-induced responses resembling those of the WT receptor. Hence, having elucidated the main trafficking characteristics of the A316T variant compared to WT GLP-1R, I next determined the coupling of each receptor with specific signalling mediators, including $G\alpha_s$, $G\alpha_i$ and β -arrestin 2 using NanoBiT complementation assays in INS-1 832/3 *Glp1r*^{-/-} cells transiently expressing the GLP-1R WT or GLP-1R A316T variant as described in section 2.2.5.

I observed a significant increase in basal mini- G_s and mini- $G_{i/o}$ coupling, with no change in β -arrestin 2 recruitment by the A316T compared to the WT receptor independent of agonist activation (Figure 4.9A-C). On the other hand, in the agonist-induced state, I found that A316T agonist-induced mini- G_s and β -arrestin 2 recruitment profiles were very similar to those of the WT GLP-1R in response to GLP-1, exendin-4, exendin-F1 or exendin-D3 (Figure 4.8D-I).

When bias between the β -arrestin 2 and mini- G_s responses was determined by calculating the difference between the two responses, I found that the A316T receptor favours $G\alpha_s$ over β -arrestin 2 recruitment only in response to exendin-F1, and that it favours β -arrestin 2 recruitment only in response to exendin-D3, relative to the wild-type receptor (Figure 4.7J).

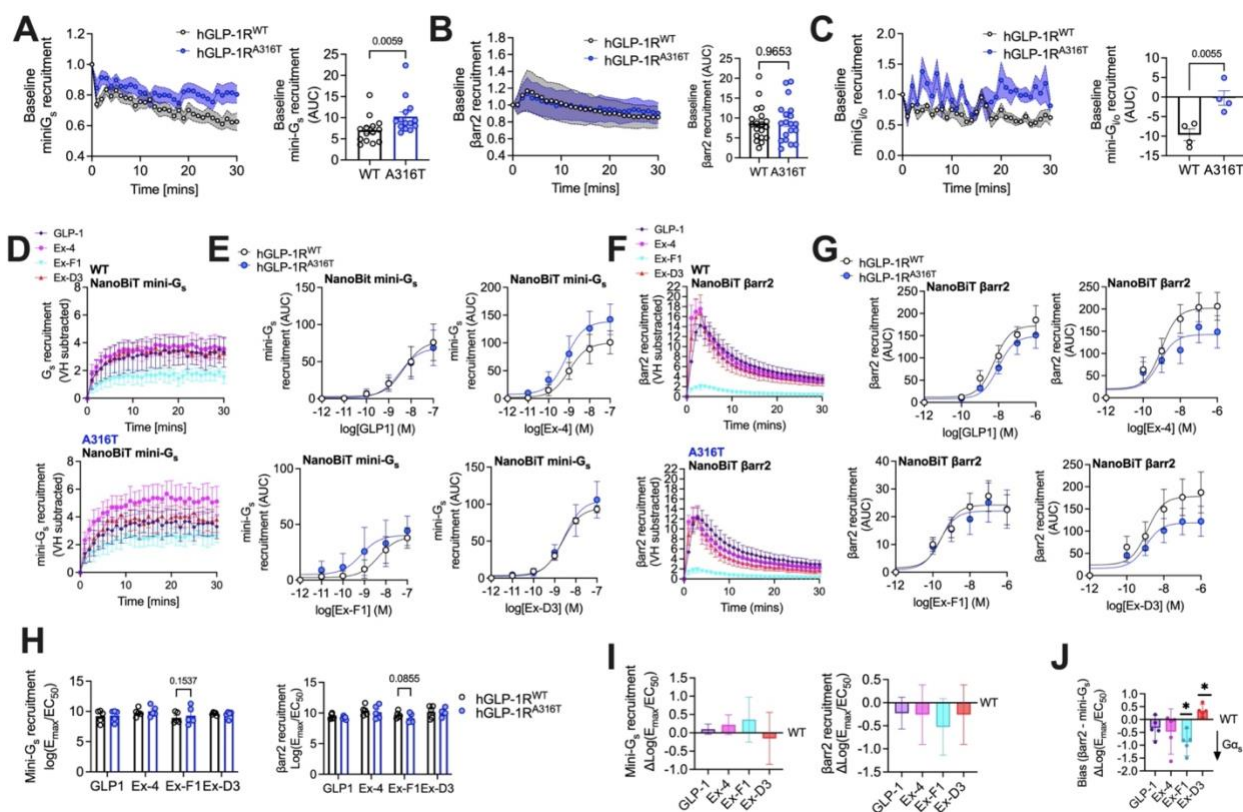


Figure 4.8 The A316T variant leads to increased basal mini- G_s recruitment and altered agonist-induced signalling profiles. **A)** Basal mini- G_s recruitment responses in INS-1 832/3 *Glp1r*^{-/-} transiently transfected with each SmBiT-tagged GLP-1R A316T or WT receptor and LgBiT-tagged effector and corresponding AUCs. **B)** Basal β -arrestin 2 recruitment responses with the same conditions as A and corresponding AUCs. **C)** Basal mini- $G_{i/o}$ recruitment responses in INS-1 832/3 *Glp1r*^{-/-} transiently transfected with each SmBiT-tagged GLP-1R A316T or WT receptor and LgBiT-tagged effector and corresponding AUCs. **D)** Agonist-induced mini- G_s recruitment responses in the same cells with 100 nM GLP-1, exendin-4 (Ex-4), exendin-F1 (Ex-F1) or exendin-D3 (Ex-D3); n=5 for each genotype and **E)** corresponding log-dose response curves. **F)** Agonist-induced β -arrestin-2 recruitment responses with the same conditions as in D) and **G)** corresponding log-dose response curves. **H)** Quantification of the effect of the A316T mutation on agonist efficacy in inducing mini- G_s and β -arrestin 2 recruitment [$\log(E_{max}/EC_{50})$] and **I)** corresponding differences to WT responses [$\Delta\log(E_{max}/EC_{50})$].

J) Calculation of receptor signalling pathway bias for each agonist, calculated from data in H and I. Comparisons of A316T *versus* WT were made with paired t-tests or two-way analysis of variance (ANOVA) with Sidak's post-hoc tests. Data are presented as means $\pm$ SEM or 95% CI.

4.2.7 Endosomal versus plasma membrane signalling of the A316T GLP-1R in agonist-free and agonist-induced states

Since the A316T GLP-1R exhibits increased basal trafficking resulting in reduced cell surface expression levels, but despite this it maintains a GoF phenotype for $G\alpha_s$ coupling, I next determined the spatiotemporal pattern of $G\alpha_s$ activation elicited by this variant receptor to elucidate if this GoF phenotype can be explained by increased $G\alpha_s$ recruitment in endosomes. To do this I employed a bystander NanoBiT signalling assay developed and described previously by our group, based on the recruitment of nanobody 37 (Nb37), which binds to activated $G\alpha_s$ - to plasma membrane *versus* endosomal locations in the presence or absence of agonist stimulation as described in section 2.2.5. While the assay showed no significant differences in the recruitment of Nb37 to the plasma membrane and endosomes by the ligand-free A316T GLP-1R compared to the WT GLP-1R (Figure 4.9A and B), there was a non-significant increase in the ratio of endosomal to plasma membrane activity with the mutant receptor (Figure 4.9C). This indicates that even though the A316T GLP-1R exhibits an intrinsic GoF, this is not explained by an increase in endosomal $G\alpha_s$ activity under basal conditions.

In terms of the agonist-dependent effects, plasma membrane and endosomal activity dose response curves of both receptors revealed ligand-dependent differences once differences in surface expression were considered, revealing that A316T GLP-1R endosomal activity is

significantly improved in response to GLP-1 and markedly decreased in response to exendin-4, whilst the response to the other agonists remains unchanged (Figure 4.9H). This increase in efficiency of A316T receptor endosomal activity in response to GLP-1 matches the internalisation profile observed in the previous trafficking studies.

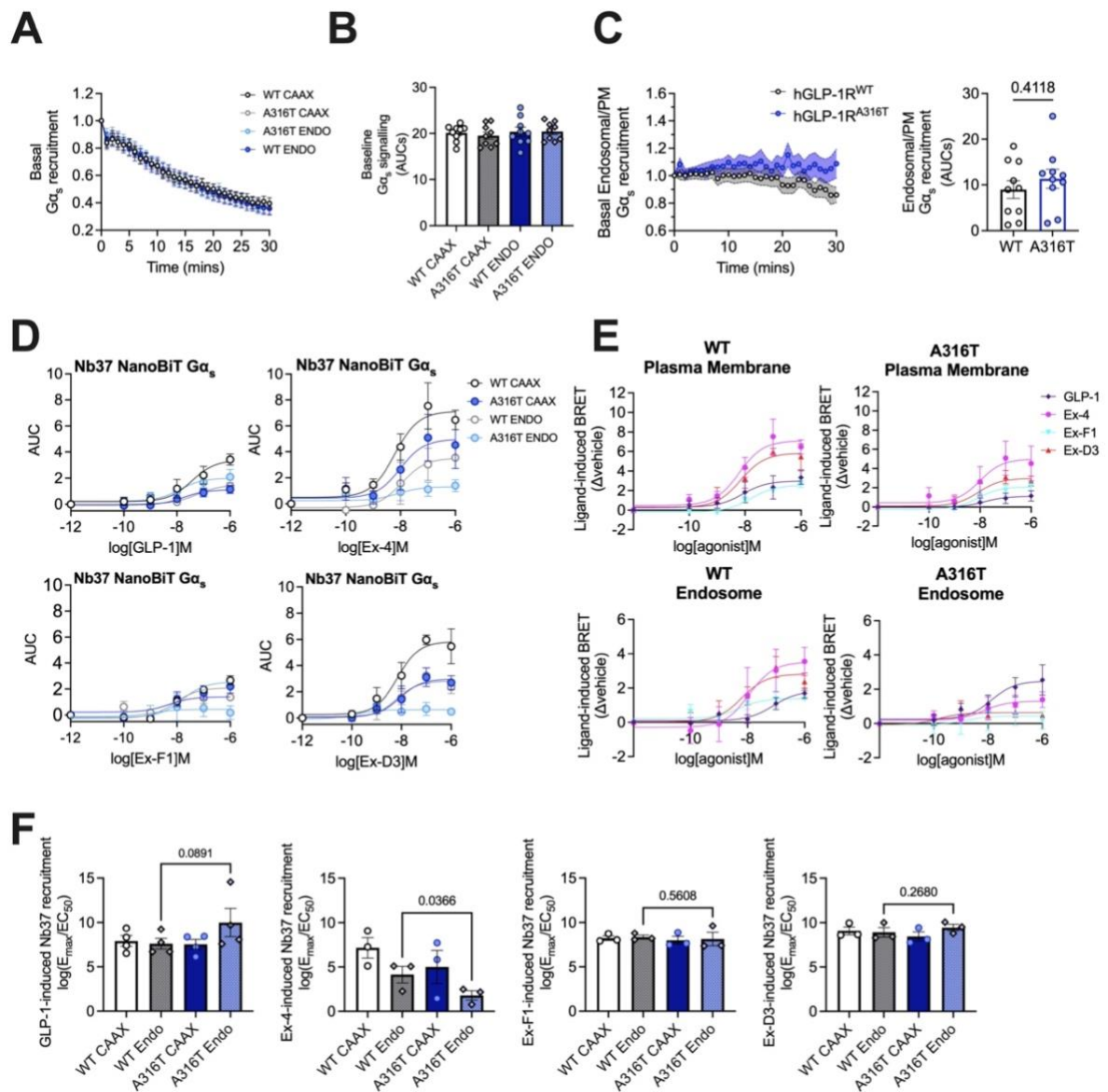


Figure 4.9 Endosomal versus plasma membrane activity of hGLP-1R WT vs A316T in rat β -cells. **A)** A316T versus WT GLP-1R endosomal (Endo) and plasma membrane (CAAX) $G\alpha_s$ recruitment responses from NanoBiT bystander assays performed in INS-1 832/3 GLP-1R $^{-/-}$ transiently expressing Nb37-SmbiT, LgBiT-CAAX and SNAP-GLP-1R WT or -A316T, in the absence of agonists and corresponding AUCs (**B**). **C)** The same as A but expressed as ratio of endosomal vs plasma membrane responses and corresponding AUCs. **D and E)** Dose-response curves from the same assays as A after stimulation with the indicated concentrations of GLP-1, Exendin-4 (Ex-4), Exendin-Phe1(Ex-F1), and Exendin-Asp3 (Ex-D3). **F)** Quantification of the effect of the A316T mutation on agonist efficacy in inducing endosomal (Endo) versus plasma membrane $G\alpha_s$ recruitment ($\log(E_{max}/EC_{50})$). Comparisons of A316T versus WT were made with paired t-tests or one-way analysis of variant (ANOVA) with Sidak's post-hoc tests. Data are presented as means $\pm$ SEM or 95% CI.

4.2.8 The A316T variant alters the agonist-induced cAMP responses of the GLP-1R

As mentioned in previous sections, the A316T variant signalling profile has been characterised by previous studies, revealing conflicting effects on downstream signalling pathways such as cAMP accumulation and Ca^{2+} mobilisation, with some studies revealing a GoF phenotype, no effect, or LoF relative to WT GLP-1R in HEK293 and CHO cells (43, 382, 527). Yet, to my knowledge, this has not been investigated in pancreatic β -cells.

Changes in GLP-1R cAMP accumulation are often a result of the combined effects of receptor trafficking and $G\alpha_s$ recruitment. Hence, I sought to determine cAMP responses of the A316T variant when stimulated with different GLP-1RAs in INS-1 832/3 *Glp1r* $^{-/-}$ β -cells stably expressing the SNAP-tagged A316T GLP-1R or WT. Despite not observing any significant differences in acute cAMP responses, I found a tendency for increased cAMP response to GLP-1 and reduced responses to exendin-4, yet no change in responses to semaglutide, exendin-F1 or exendin-D3 in the A316T compared to WT receptor (Figure 4.10A, F and G).

I next assessed the level of cAMP signalling specifically from the endosome using time-lapse FRET microscopy with a ^TEpac^{VV}-based cAMP biosensor modified to localise specifically to the endosomal compartments. In line with previous findings from the Nb37 bystander assay, quantification of cAMP endosomal production after GLP-1 stimulation with the endosomal-targeted ^TEpac^{VV} biosensor demonstrated a non-significant increase in endosomal cAMP activity in the INS-1 832/3 SNAP-GLP-1R^{A316T} compared to the ^{WT} cells (Figure 4.10H and I). On the other hand, no significant change was observed in response to exendin-4 stimulation (Figure 4.10 I).

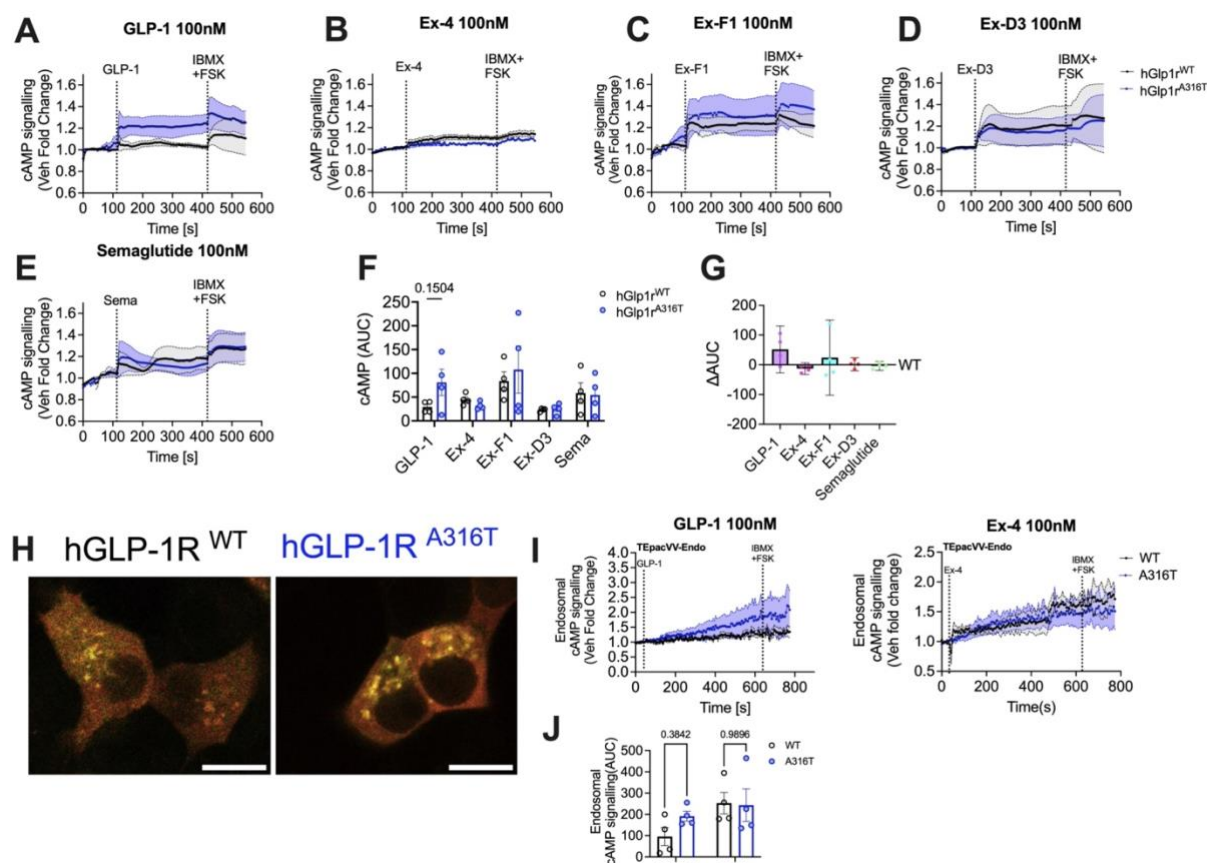


Figure 4.10 Analysis of total and endosomal cAMP signalling responses in WT and A316T hGLP-1R expressing rat pancreatic β cells. (A-E) cADD is cAMP sensor signal measured over time in INS-1 832/3 *Glp1r*^{-/-} expressing WT (black) and A316T mutant (blue) hGLP-1R in response to 100nM GLP-1 (A), Ex-4 (B), Ex-F1 (C), Ex-D3 (D), and Semaglutide (E); n=3 experiments per condition. (F) Bar graph representing the area under

the curve of cAMP responses for each ligand, comparing WT and A316T mutant receptors, and the calculated differences to wild-type response (Δ AUCs) in (G). **H**) Representative confocal microscopy images of merged CFP and YFP channels for INS-1 832/3 SNAP-hGLP-1R^{WT} or -^{A316T} cells transfected with endosome-targeted ^TEpac^{VV} cAMP biosensor construct. **I**) Top: Changes in FRET (CFP/YFP) over time in INS-1 832/3 SNAP-hGLP-1R^{A316T} vs ^{WT} control cells expressing endosome-targeted ^TEpac^{VV} cAMP biosensor constructs in response to 100 nM GLP-1 (left) and exendin-4 (right), followed by 100 μ M IBMX + 10 μ M forskolin (n=4). Bottom: AUCs calculated for the agonist treatment period. Comparisons of A316T versus WT were made with paired t-tests, Tukey's multiple comparisons or two-way analysis of variant (ANOVA) with Sidak's post-hoc tests. Data are presented as means $\pm$ 95% CIs.

4.2.9 The A316T variant does not alter the agonist-induced intracellular calcium mobilisation of the GLP-1R

Another pathway thought to be altered as a result of structural changes associated with GLP-1R variants is intracellular Ca²⁺ mobilisation. As mentioned previously, previous studies by Gao *et al.* 2023, highlight the A316T variant as a mutation that promotes a GoF phenotype for intracellular Ca²⁺ mobilization in response to stimulation with 100 nM of exendin-4 in HEK293 cells. Moreover, intracellular Ca²⁺ mobilisation is another downstream signalling readout of GLP-1R activation, controlled by G α_s and G α_q coupling.

Since I observed in the previous experiments that the A316T variant under basal conditions promotes a GoF phenotype for G α_s , resembling that of an activated wild-type hGLP-1R, but that agonist stimulation leads to only minor non-significant changes in mutant receptor responses, I predicted a similar mild phenotype with minimal differences on agonist-induced Ca²⁺ mobilization compared to the wild-type.

I used the calcium dye Cal-520AM and time-lapse fluorescence microscopy to investigate acute intracellular Ca²⁺ mobilisation in response to stimulation by GLP-1RAs and the antagonist exendin-9 in INS-1 832/3 *Glp1r*^{-/-} cells exogenously expressing SNAP-GLP-1R^{WT} or ^{A316T}. As predicted, both types of cells displayed similar calcium dynamics in response to

exendin-F1 and exendin-4 at 6 and 11mM glucose (physiological levels) (Figure 4.11B,C and D), but a non-significant increase in A316T GLP-1R calcium responses to the endogenous agonist GLP-1 and the antagonist exendin-9 (Figure 4.11A and B), which in my case was used as a proxy of the inactive-state A316T GLP-1R. Upon calculating ΔAUC (difference from wild-type response), no change was observed for any of the agonists at either glucose concentrations *versus* the WT receptor (Figure 4.11 E and F). A limitation of this calcium mobilisation assay is the use of a single agonist concentration (100nM), which may have been insufficient to elicit a detectable response, particularly in the A316T variant. The lack of a full dose-response curve limits the ability to assess the potency and efficacy of the ligands and impairs interpretation of potential differences in calcium signalling between WT and A316T mutant receptors.

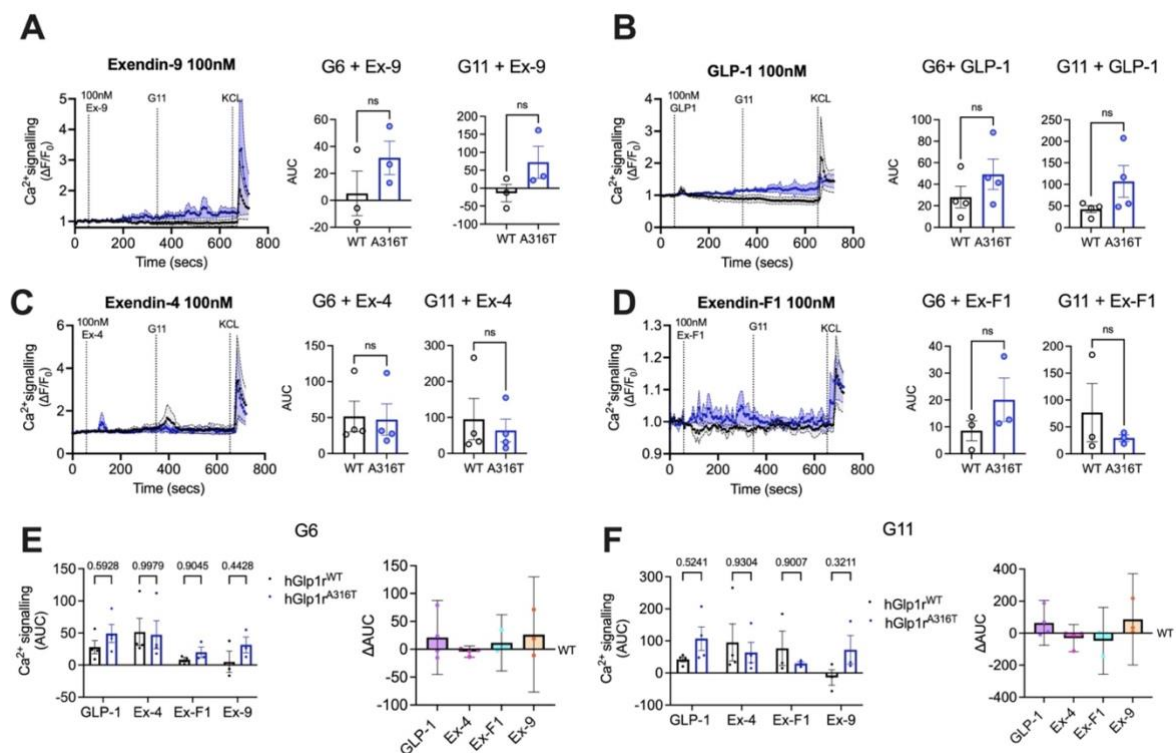


Figure 4.11 The A316T mutation leads to differential agonist-induced downstream Ca^{2+} mobilisation in rat pancreatic β cells. A) Intracellular Ca^{2+} traces measured over time in INS-1 832/3 *Glp1r*^{-/-} expressing WT

(black) and A316T mutant hGLP-1R in response to 100 nM exendin-9 (**A**), GLP-1 (**B**), exendin-4 (**C**), and exendin-F1 (**D**) at 6 mM (G6) and 11 mM (G11) glucose and corresponding AUCs. **G-H**) Comparison of variant-specific agonist responses by calculating difference of A316T and WT AUCs at 6 mM (G6) (**E**) and 11 mM (G11) (**F**) glucose concentrations. Comparisons of A316T versus WT were made with Tukey's multiple comparison test analysis with Sidak's post-hoc tests. Data are presented as means $\pm$ 95% CIs.

4.2.10 The A316T variant alters the GLP-1R interactome in rodent β -cells

To better understand the mechanisms that control the insulinotropic actions of GLP-1R, affinity purification followed by mass spectrometry (AP-MS) were employed recently by my group (540) to uncover potential proteins that differentially interact with the GLP-1R in response to different GLP-1RAs such as GLP-1, exendin-4 and the biased exendin-F1 and exendin-D3. AP-MS performed on the rat pancreatic β -cell line INS-1 832/3 *Glp1r*^{-/-} stably expressing SNAP-hGLP-1R, revealed over 80 proteins with potential changes in their association with the GLP-1R in active *versus* inactive conditions (540). Two novel GLP-1R interactors [VAMP associated protein B (VAPB) and the A-kinase anchoring protein SPHKAP] were further validated experimentally to reveal a crucial role for GLP-1R signalling at ER membrane contact sites (MCS), enabling GLP-1R-induced mitochondrial remodelling and maintenance of optimal β -cell function (540).

Since interactions with these 80 proteins are enhanced in active *versus* inactive WT hGLP-1R, I expected the A316T variant to exhibit enhanced interactions with these proteins in the basal state. On the other hand, as with other trafficking and signalling profiles, agonist-induced A316T GLP-1R interactions may exhibit a different pattern compared to the WT receptor. Hence, I sought to elucidate the A316T GLP-1R interactome profile in basal and following stimulation with GLP-1 and exendin-4, utilising the same established anti-Flag affinity purification and LC-MS/MS analysis method as used in previous study, on INS-1 832/3 *Glp1r*

^{-/-} cells expressing the SNAP-hGLP-1R A316T and compared interactors obtained from this study with those previously observed for the WT GLP-1R.

Expectedly, the ligand-free A316T GLP-1R exhibited markedly increased interactions with previous WT GLP-1R interactors, suggesting an intrinsic GoF phenotype for this variant compared to the WT, in line with previous findings (Figure 4.12A). Encouragingly, these interactors included known active GLP-1R binding proteins such as G α_s , AP2, Rab5 and β -arrestin 2 (*Arrb2*). The increased G α_s interaction validated my findings from NanoBiT experiments. There are a few interactors, such as the ubiquitin-like protein SUMO1, which show reduced interaction in active *versus* inactive WT GLP-1R. Conversely, the ligand-free GLP-1R A316T variant exhibits increased interaction with SUMO1, which gets further increased for the variant receptor following agonist stimulation, suggesting that this mutation may activate distinct pathways in addition to enhancing existing ones (Figure 4.12A).

In parallel to this, other differences between the A316T and WT GLP-1R were also observed specifically in the agonist-induced interactome. Strikingly, I identified 22 potential interactors that exhibit decreased association with the A316T GLP-1R upon agonist stimulation, contrary to the WT GLP-1R (Figure 4.12B). Noteworthy, these included the previously investigated SPHKAP and VAPB, suggesting changes in the capacity for A316T GLP-1R to potentiate specific signalling nanodomains that might regulate pancreatic β -cell function and insulin secretion compared to the WT receptor.

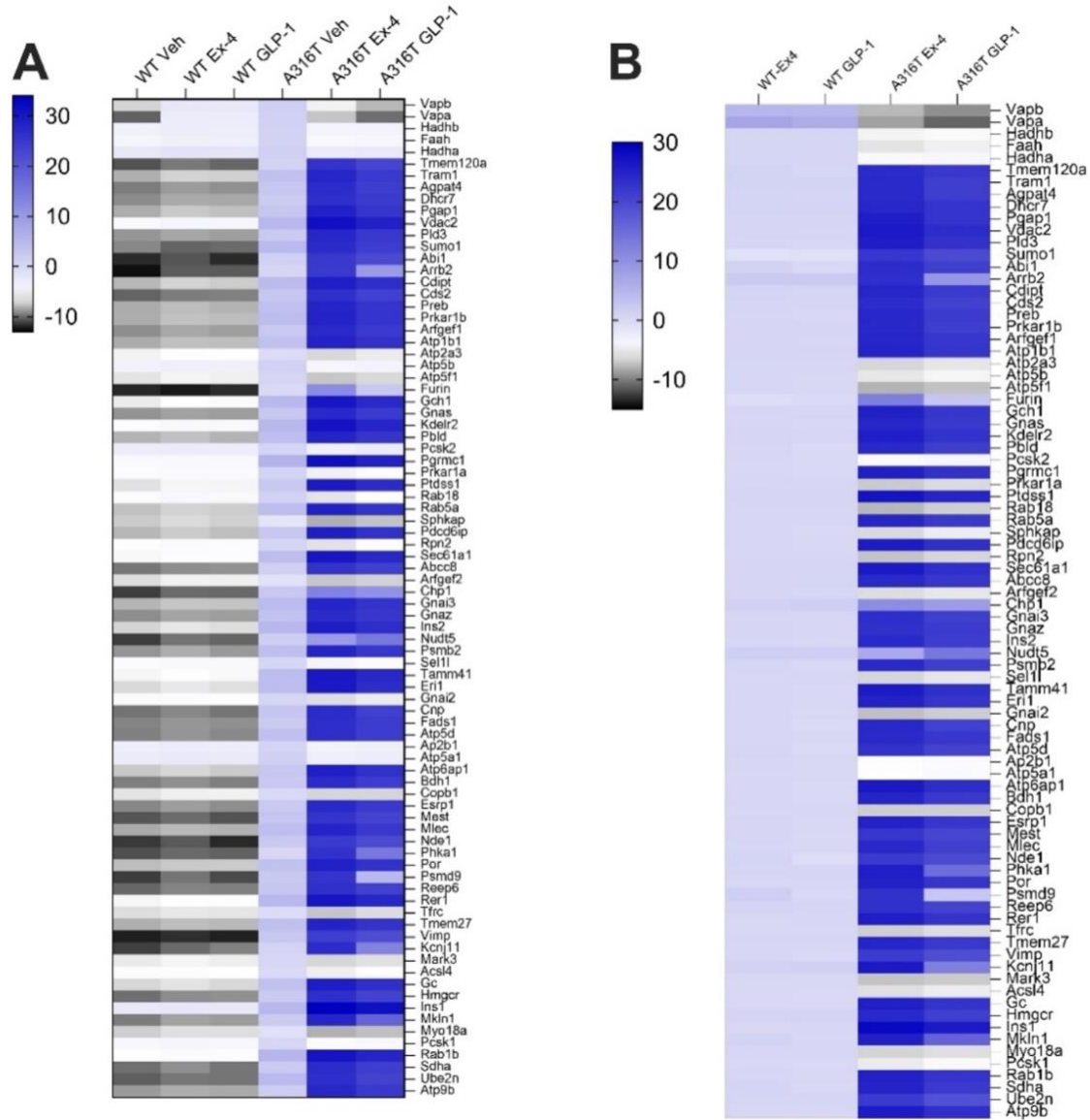


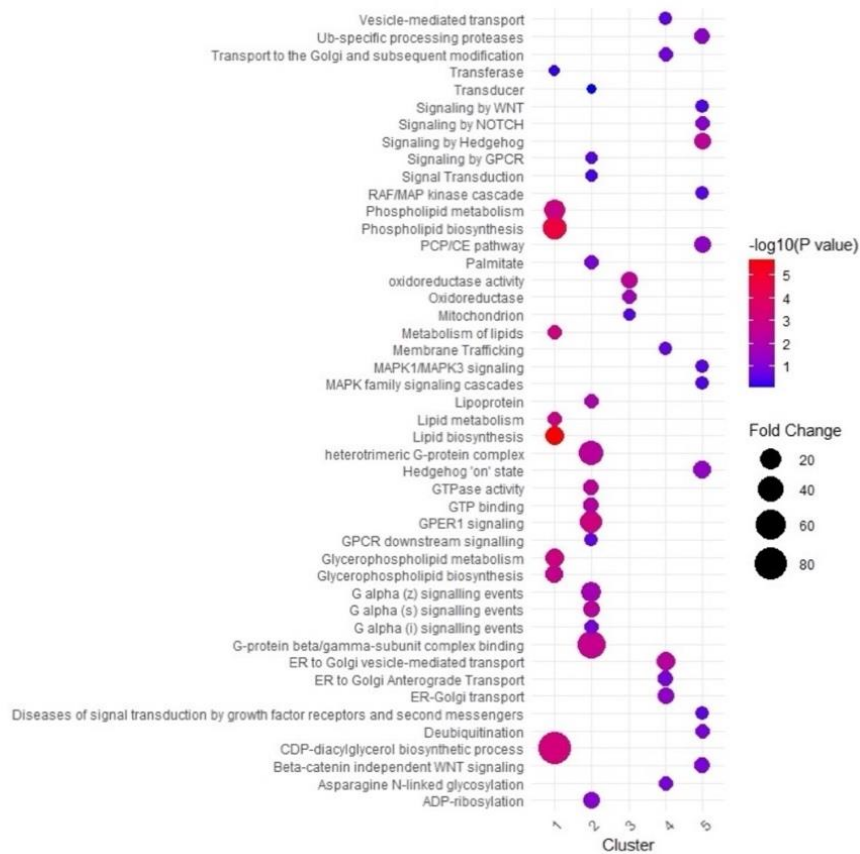
Figure 4.12 The A316T variant-dependent changes in the GLP-1R interactome. A) Heatmap of A316T GLP-1R interactor enrichment from anti-FLAG co-immunoprecipitates from (VH) versus 5-minute exendin-4 (Ex-4) or GLP-1-stimulated INS-1 832/3 SNAP-hGLP-1R A316T and WT expressing cells; black, decreased; blue, increased enrichment. **(B)** Heatmap comparing difference in interaction of the A316T variant versus the WT in response to exendin-4 and GLP-1 (difference from own vehicle). LC-MS/MS data analysed in LFQ-Analyst and normalised to hGLP-1R levels for each experimental sample; colour scale centred around overall median value; n=3.

To assess the effect of the A316T variant on the agonist-induced interactome, all interactors with changes in A316T GLP-1R association *versus* basal conditions were uploaded and analysed by DAVID to produce gene clusters. From the provided annotation categories, I selected GO, UniProt and KEGG pathway. I used the Functional Annotation Clustering tool, which organises annotation terms into simplified clusters by merging similar terms within the group. A total of 9 gene clusters were produced, and I took the clusters with the highest enrichment scores, revealing functional annotations which, for the case of factors enriched in their interaction with the A316T GLP-1R under stimulated conditions, include phospholipid metabolism, glycerophospholipid biosynthesis, $G\alpha_s$ signalling, and ER to Golgi transport.

On the other hand, gene ontology analysis of interactors with reduced association to the A316T receptor in response to GLP-1 or exendin-4, revealed functional annotations for fatty acid degradation and metabolism pathways (Figure 4.13 A and B). Due to the time and cost constraints of this study, further assessment of these diminished interactions was not performed. Therefore, future studies should validate these altered interactions in the same cells to investigate the underlying reason behind their reduced association with the mutant receptor in response to agonist stimulation, and thus if this is detrimental or beneficial to pancreatic β -cell function.

A

Gene Ontology Analysis Enriched Associations



B

Gene Ontology Analysis Diminished Associations

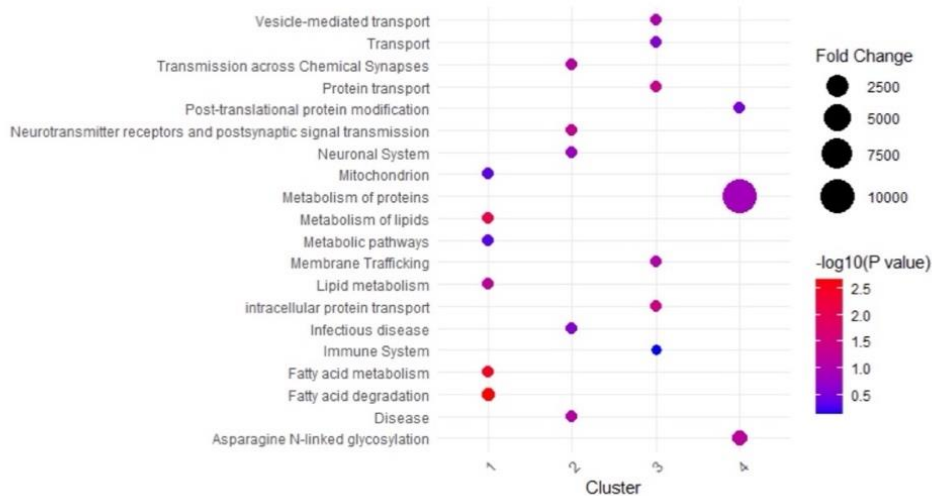


Figure 4.13 Gene Ontology (GO) analysis of enriched and diminished associations in WT and A316T hGLP-1R expressing rat pancreatic β cells. A) GO analysis of enriched associations in INS-1 832/3 SNAP-hGLP-1R A316T compared to WT expressing cells. **B)** Gene Ontology (GO) analysis of diminished associations in the same cells. The dot plot represents various biological processes with significant enrichment or diminution. The dot size indicates fold change, while the colour gradient represents the $-\log_{10}$ (P value) with red indicating higher significance. The clusters represent distinct biological processes or pathways identified through the analysis. Dot blots were generated in R software with the ggplot2 package and enrichments were computed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) server.

4.2.11 The A316T variant alters GLP-1R ubiquitination patterns

Ubiquitination is a post-translational modification where ubiquitin, a small regulatory protein, is attached to a protein such as a GPCR, influencing a variety of cellular processes. When it comes to class B1 GPCRs like the GLP-1R, ubiquitination is a critical regulatory mechanism, influencing receptor internalisation, trafficking, stability and signalling functions (595, 596). Ubiquitination serves as an internalisation signal, whereby canonically following activation of the GPCR by ligand binding it is tagged with ubiquitin, sorting and trafficking it within the endosomal-lysosomal system, thus removing the receptor from the cell surface and aiding in the desensitisation of the cell to further stimuli (595, 596). For instance, activity of GIPR, the other incretin receptor, is down-regulated in a glucose dependent manner by ubiquitination in pancreatic islets (597). As for GLP-1R, a previous RNAi screen by our group which unearthed novel factors involved in regulation of exendin-4 induced insulin secretion in pancreatic β -cells as well as recent studies of the GLP-1R interactome in rodent pancreatic β -cells, identified NEDD4, a mediator of β -arrestin 2 dependent ubiquitination, as an important GLP-1R interactor that is involved in regulating exendin-4 stimulated insulin secretion (361). Building on this finding, further studies by our group revealed that the GLP-1R is

constitutively ubiquitinated and associated with NEDD4 in the absence of an agonist and undergoes limited deubiquitination upon agonist activation (362).

Consequently, having established the trafficking profile of the A316T GLP-1R, including increased lysosomal trafficking, I investigated the degree of ubiquitination of this mutant receptor as a potential mechanism to explain its intrinsic increased lysosomal trafficking. For this, I performed GLP-1R immunoprecipitation experiments in INS-1 832/3 *Glp1r*^{-/-} stably expressing SNAP-hGLP-1R A316T *versus* WT. Consistent with our previous studies (362), results showed that both the WT and A316T receptor were ubiquitinated in vehicle conditions (Figure 4.14A). Yet, while the WT GLP-1R underwent partial de-ubiquitination following exendin-4 stimulation, I observed a significant increase in the ubiquitination of the mutant A316T GLP-1R in response to exendin-4 stimulation (Figure 4.14C).

As mentioned previously, ubiquitination is associated with receptor lysosomal targeting, so this increase might be related to changes in lysosomal targeting of the mutant receptor.

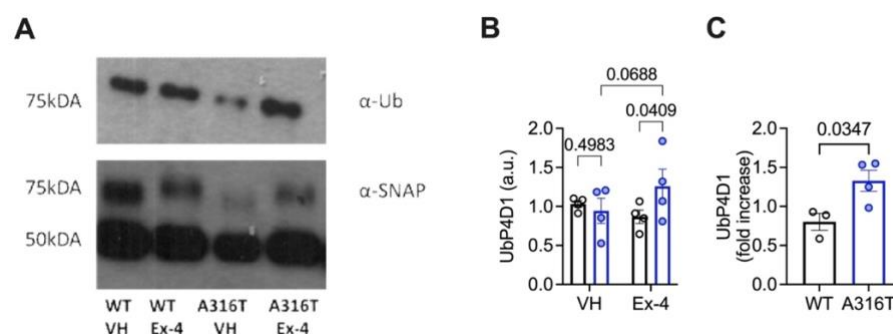


Figure 4.14 The A316T GLP-1R exhibits altered ubiquitination in rat pancreatic β -cells. A) Representative blots showing SNAP-hGLP-1R ubiquitination levels following SNAP-hGLP-1R immunoprecipitation from INS-1 832/3 SNAP-hGLP1R A316T vs WT cells under vehicle conditions or after stimulation with 100 nM GLP-1 or exendin-4 (Ex-4) for 10 minutes, with quantification of ubiquitin over SNAP levels for the different conditions shown (B); n=5. C) Quantification of fold increase compared to vehicle. Comparisons of A316T *versus* WT were made with paired t-tests or two-way analysis of variance (ANOVA) with Sidak's post-hoc tests. Data are presented as means $\pm$ SEM.

4.2.12 Characterisation of the A316T variant in a human β -cell model

Whilst the INS-1 832/3 rat insulinoma cells are a robust model widely used to study GLP-1R action in response to different agonists as well as GLP-1R signalling and trafficking, these cells may not fully recapitulate gene expression and functionality of human β -cells. In addition to this, since we are using a system that overexpresses the human GLP-1R, cross-species differences in receptor isoforms and the presence of rodent-specific pathways might mean that the effects observed are less relevant to human receptor function.

Therefore, I generated a GLP-1R knock-out enriched pool of immortalised human EndoC- β H3 β -cells using a dual lentiviral CRISPR/Cas9 sgRNA strategy as described in section 2.2.17, and subsequently performed functional characterisation in these cells following transduction with adenoviral vectors expressing SNAP-hGLP-1R^{WT} or A316T. Although, previous studies showed that the EndoC- β H3 cell line exhibits markedly impaired *Glp1r* expression compared to primary human β -cells and compared to the more recently developed EndoC- β H5 cells (Figure 4.15A) (591), I still proceeded with the CRISPR/Cas9 knock-out in these cells to ensure the elimination of background noise caused by any residual endogenous GLP-1R, so that any observed effects can be attributed specifically to the exogenously expressed SNAP-tagged WT or A316T mutant receptors.

The RNA-guided Cas9 nuclease from the microbial clustered regularly interspaced short palindromic repeats (CRISPR) adaptive immune system is used to facilitate efficient knock-out of genes in mammalian cells by simply specifying a 20-nt targeting sequence within its guide RNA (gRNA) (598). This gRNA guides the nuclease to the target locus where it cleaves generating double strand breaks (DSBs), these DSBs then undergo one of the two major

pathways for DNA damage repair: the error-prone non-homologous end joining (NHEJ) or the high-fidelity homology directed-repair (HDR) (598). In cases like mine, where a repair template is not provided, DSBs are re-ligated through the NHEJ process, leaving insertion/deletion (indel) mutations within coding exons resulting in frameshift mutations and premature stop codons which ultimately lead to gene knockouts (598).

For this study, I used a modified dual lentiviral CRISPR/Cas9 approach, previously demonstrated to robustly generate KOs in genes crucial for β -cell function including insulin (INS) and peptidyl-glycine alpha-amidating monooxygenase (PAM) (546, 599, 600). This well-established lentiviral-based pipeline was used here due to known technical challenges of using this cell line including its very low transfection efficiency, their low proliferation rate and their sensitivity to seeding densities. The lentiCRISPRv2-Blast vector system is a one-plasmid system containing Cas9, a blasticidin resistance cassette and the cloned sgRNAs. This specific plasmid with a blasticidin resistance cassette was chosen as the EndoC- β H3 already contains both a CRE-ERT2 and constitutive puromycin selection marker integrated by lentiviral-mediated gene transfer. Briefly, EndoC- β H3 cells were transduced with a Cas9-expressing lentivirus containing two sgRNAs designed to selectively target two different loci in exon 2 of the GLP-1R. Successfully transduced cells were then selected for with 10 mg/ml blasticidin, followed by reverse FACS sorting via labelling the remaining GLP-1R expressing cells with the fluorescent agonist exendin-9-TMR, and thus eliminating them from the remaining pool of cells. Lastly, I characterised the generated heterogenous GLP-1R knock-out cell line with fluorescent agonist labelling and confocal microscopy to assess endogenous GLP-1R levels as well as functional studies to assess LoF (Figure 2.7).

Functional characterisation of EndoC-βH3 GLP-1R Knock-out cells

To determine if the genomic modifications resulting from stable lentiCRISPRv2 integration and CRISPR editing translate into functional GLP-1R KO cells, I investigated cAMP production using a HTRF assay, one of the main downstream signalling pathways engaged by the GLP-1R after agonist binding, in comparison to unedited EndoC-βH3 cells. I determined if response to agonists and resulting downstream signalling pathways, such as cAMP signalling, were also reduced indicating a successful functional knock-out. I observed that the EndoC-βH3 GLP-1R knock-out cell line exhibited significant reductions in cAMP levels in the absence of agonist, as well as in response to the GLP-1R agonist exendin-4 and forskolin, a well-established activator of the enzyme AC responsible for converting ATP to cyclic AMP and rapidly increasing intracellular cAMP levels, compared to the wild-type control cell line (Figure 4.15B).

This reduction in intracellular cAMP levels, and in response to the GLP-1R agonist exendin-4 indicates that the created subline is GLP-1R knock-out. However, I cannot rule out the presence of a heterogeneous cell population as single clones were not selected with the required targeted locus. This is mainly due to technical difficulties pertaining to their very low proliferation rate especially at low seeding densities, such as these needed to expand a culture from a single cell. Albeit, for my studies I was satisfied with this functional knock-out, as the main comparisons I am interested in are between the mutant and the wild-type receptor, which was exogenously expressed via an adenoviral system.

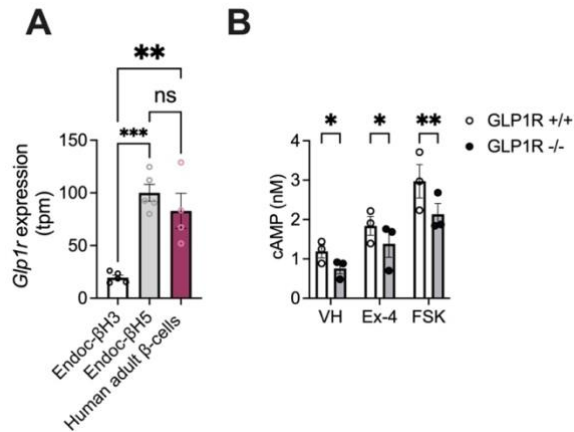


Figure 4.15 Characterisation of the EndoC-βH3 GLP-1R^{-/-} cell line. **A)** Quantification of *Glp1r* gene expression (tpm) from EndoC-βH3, EndoC-βH5, and FACS sorted β-cells from primary human β cells; data was obtained from a published RNA-Seq database by Blanchi *et al.* (2023). **B)** cAMP production in response to vehicle, 100 nM exendin-4 (Ex-4) and Forskolin (FSK) in EndoC-βH3 GLP-1R^{-/-} versus EndoC-βH3 control cells as assessed by HTRF assay (n=3). Comparisons of A316T versus WT were made with paired t-tests or two-way analysis of variant (ANOVA) with Sidak's post-hoc tests. Data are presented as means ± SEM. Scale bars indicate 50 μm.

The A316T variant reduces membrane localisation of the GLP-1R in EndoC-βH3 cells

Given the significantly impaired cell surface expression of the A316T variant in previous models investigated, including the HEK293 cells and CHO cells by others (382, 518), as well as the INS-1 832/3 rat pancreatic β-cells in this thesis, I sought to determine if the same A316T phenotype would be seen when the human GLP-1R A316T variant is overexpressed in pancreatic β-cells of human rather than rodent origin. I performed these experiments utilising the EndoC-βH3 GLP-1R knock-out cell line I generated and characterised in the preceding section. I then used an adenovirus system outsourced from Vectorbuilder, as it is technically challenging, resource-intensive, and time consuming to produce such high-titer and high-quality viral preparations in the laboratory by ourselves. I provided the company with the plasmid vectors described in section 2.1.3 pSNAP-*hGLP-1R*^{WT} and ^{A316T} to be used for the assembly of the adenoviral vector plasmid and subsequent packaging into live adenoviruses.

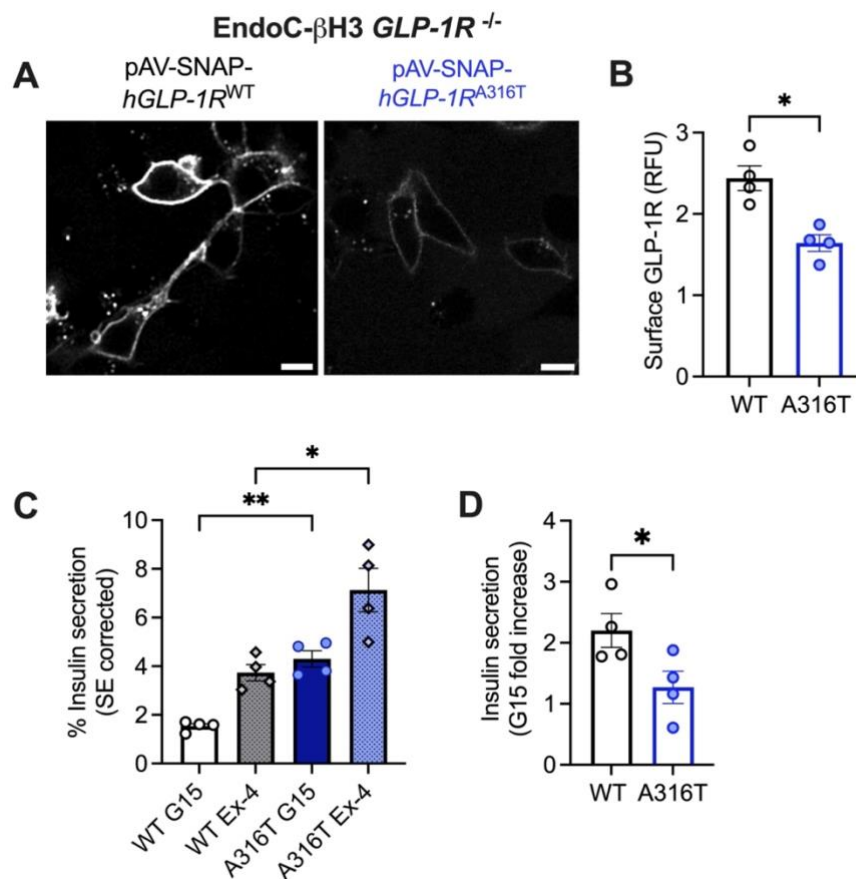
An adenovirus system was used to overexpress these constructs as previous attempts using lentivirus vectors produced *in-house* only resulted in successful transduction of HEK293 and INS-1 832/3 cell lines but not the more challenging EndoC- β H3 cell line. Adenoviruses have been shown to infect a wide variety of cell types with high transduction efficiency, and they have larger genomic capacities compared to other viral systems (such as lentiviruses) allowing for the incorporation of larger genes, sequences, and elements required for optimal receptor expression.

To compare A316T *versus* WT GLP-1R expression in EndoC- β H3 cells, I first transduced the cells with the corresponding adenoviral vectors at an MOI of 1 for 24 hours, followed by labelling with a fluorescent SNAP-probe for 10 minutes and imaging with confocal microscopy. Expectedly, I found that even in cells of a human origin the A316T GLP-1R is exhibiting a significant reduction in cell surface expression (Figure 4.16A and B). This is unlikely to be due to the adenoviral vector system used as these vectors often utilize strong viral promoters, in my case the CMV promoter for both receptor genotypes, driving high levels of gene expression. This validates the phenotype along with increased basal trafficking which is present regardless of the vector or cell model used to overexpress the mutant receptor.

The A316T variant enhances GSIS in EndoC- β H3 cells

Subsequently, to assess the functional consequences of the A316T GLP-1R variant in a physiologically relevant model compared to the INS-1 832/3 cells, I then measured incretin-dependent and independent GSIS using HTRF assays in the EndoC- β H3 (*GLP-1R* KO) cells from above. Expression of the A316T GLP-1R showed a non-significant small increase in GSIS both in the absence and presence of the GLP-1RA exendin-4 compared to the WT GLP-

1R (Figure 4.16C). I next accounted for differences in cell surface expression levels to ensure that observed changes in insulin secretion are due to differences in receptor function and signalling rather than receptor presence on the cell surface, allowing for a more accurate comparison. The enhanced GSIS phenotype observed in A316T-expressing cells was now more pronounced reaching significance upon accounting for the reduced receptor expression (Figure 4.16D). GSIS responses specifically elicited by the variant relative to the WT receptor in the agonist stimulated state were determined as fold increases from 15 mM glucose responses for each of the genotypes, observing that responses were significantly reduced in the A316T GLP-1R compared to WT GLP-1R expressing cells (Figure 4.16E).



1R^{-/-} cells transduced with pAV-SNAP-hGLP-1R^{WT} or ^{-A316T} adenoviruses and labelled with SNAP-Surface Alexa Fluor 647 for 10 minutes. Fluorescence for SNAP-hGLP-1R is shown (white), and bars indicate 20µm. **B)** Quantification of hGLP-1R surface expression from A. **C)** Effect of the A316T mutation on insulin secretion in the presence of 15 mM glucose (G15) only, or with exendin-4 (Ex-4) treatment. **D)** Values in C corrected for surface expression values. **E)** Normalised % insulin secretion values demonstrating the effect of Ex-4 versus G15 alone, calculated from C. Comparisons of A316T *versus* WT were made with paired t-tests or two-way analysis of variant (ANOVA) with Sidak's post-hoc tests. Data are presented as means ± SEM.

4.3 Discussion

The aim of the work described in this chapter was to characterise the *in vitro* effects of the A316T variant on GLP-1R function utilising two distinct pancreatic β-cell lines: the rat insulinoma INS-1 832/3 pancreatic β-cell line and the human EndoC-βH3 cell line, both immortalised cell lines. Understanding the functional implications of this variant in these model systems is critical for elucidating the underlying mechanisms behind its beneficial glycaemic phenotype. As such, experiments performed in this chapter provide detailed insights into how the A316T variant affects GLP-1R trafficking, intracellular localisation, signal transduction, protein-protein interactions, and downstream signalling including Ca²⁺ mobilisation and cyclic AMP production, plus insulin secretion in these immortalised cell lines, thereby contributing to a broader understanding of the molecular mechanisms underlying A316T GLP-1R function in more physiologically relevant model systems compared to previous studies.

I built upon previous research findings on the A316T variant done in non-pancreatic cell lines like HEK293 and CHO (382, 518, 601), which identified key phenotypic characteristics such as significantly decreased cell surface expression, enhanced agonist-induced mini-G_s recruitment, and unchanged or reduced responses to exendin-4, culminating in an overall GoF

phenotypic profile. My selected cell lines offer the advantage of maintaining the native cellular context, including relevant signalling pathways and receptor-protein interactions inherent to pancreatic β -cells, thereby enhancing the physiological relevance of the findings. Additionally, these cell lines are readily available and well-suited for high-throughput screening of therapeutic compounds, signalling studies, and other large-scale assays that would be impractical with other cell types. Thus, they provide an optimal balance of physiological relevance and experimental convenience to study my chosen polymorphism.

4.3.1 Influence of the A316T variant on GLP-1R function in rodent INS-1 832/3 cells

Effect of the A316T variant on cell surface expression

Cell surface expression of GPCRs such as the GLP-1R is essential for effective ligand binding and signal transduction, enabling efficient cellular responses to external stimuli. It also influences pharmacodynamics and pharmacokinetics, as drugs need accessible receptors to function properly. GLP-1R expression is regulated by receptor turnover rate, trafficking and biosynthesis. In fact, Gao *et al.* (2023), highlights that severely impaired cell surface expression in GLP-1R variants contributes to impaired glucose control and increased adiposity (601). Paradoxically, despite its defective cell surface expression, the A316T variant exhibits a GoF phenotype for GLP-1R function. Consistent with previous studies in HEK293T and CHO cell lines (382, 518, 601), I also observed a significant reduction in cell surface expression of the A316T GLP-1R in comparison to the wild-type, in cells transiently and stably expressing SNAP-GLP-1R^{A316T}. I used an overexpression system, whereby SNAP-GLP-1R is expressed heterologously via a plasmid vector. Thus, I can be confident that as the

receptor is being expressed under a CMV promoter, this will ensure robust transcription and high levels of receptor biosynthesis. The reduction in cell surface expression is therefore more likely due to post-translational mechanisms, such as improper folding, retention and degradation of the mutant GLP-1R in the ER, defective trafficking preventing the receptor from reaching the plasma membrane, and/or enhanced degradation by cellular quality control mechanisms like the ubiquitin-proteasome system, or disrupted glycosylation, or alternatively increased basal turnover and lysosomal degradation.

Effect of the A316T variant on trafficking

As impaired receptor trafficking, including enhanced degradation and endocytosis are key reasons for reduced cell surface expression and impaired plasma membrane localisation, I have compared WT and A316T receptor trafficking utilising high-content microscopy and confocal microscopy techniques, in conjunction with SNAP-tag probes and fluorescent GLP-1R agonists. In examining the mechanisms responsible for the reduced cell surface expression of the A316T variant in INS-1 832/3 cells, I used the lysosomal inhibitor bafilomycin to answer this question. Bafilomycin A1 treatment completely rescued the cell surface expression levels of the A316T receptor to WT receptor levels. This finding indicates that the impaired cell surface expression of this variant is due to an increased rate of basal lysosomal trafficking and a higher rate of receptor turnover. Studies on other WT and mutant GPCRs have shown that partially active receptor conformations can form even in the absence of a ligand, leading to constitutive, ligand-independent signalling (602). The intermediate conformational state (State 1) adopted by the ligand free A316T GLP-1R likely confers a partially active phenotype, leading to its increased receptor turnover and degradation via lysosomal pathways. The intermediate conformational state adopted by the A316T could allow for more frequent

endocytosis due to increased basal signalling activity and recruitment of endocytic proteins, as seen in other GPCRs with specific mutations affecting receptor conformation and trafficking (603, 604). I have also assessed the internalisation, recycling and trafficking characteristics of the A316T GLP-1R in response to various agonists, including GLP-1, exendin-4, exendin-F1, and exendin-D3. The results demonstrated that the A316T variant exhibited slightly different trafficking profiles depending on the agonist used. In terms of internalisation, the A316T variant exhibited significantly increased internalisation only in response to GLP-1. The endogenous agonist also shows significantly reduced recycling rates. There are also non-significant increases in receptor degradation rates to all agonists apart from exendin-F1. While it is difficult to speculate the exact reasons for the differences encountered, they seem to indicate a differential interaction of the A316T receptor with endogenous GLP-1 compared with exendin-4-derived compounds.

I have also explored here the propensity for the mutant receptor to localise to membrane raft nanodomains as this is linked to GLP-1R activation and endocytosis (358). Consistent with the increased basal trafficking profile observed for this receptor mimicking a ligand-activated WT receptor, the A316T GLP-1R exhibited increased basal recruitment into membrane raft nanodomains. Upon stimulation with GLP-1, both receptors showed modest increases in nanodomain partitioning. However, exendin-4 treatment resulted in decreased partitioning into membrane nanodomains for the A316T variant compared to the WT, again indicating differences in receptor-ligand interactions between the GLP-1 and exendin-4 bound states. Receptors recruited to membrane nanodomains are often more prone to form homodimers, i.e. clustering (358). Nonetheless, TR-FRET analysis revealed no significant change in receptor clustering for the A316T variant under both vehicle and GLP-1-stimulated conditions. This

lack of observed differences in clustering could be due to the limited sensitivity of the TR-FRET technique. More sensitive approaches, such as RICS provide higher resolution and more accurate spatial and temporal information about mutant receptor clustering and dynamics within the plasma membrane. RICS experiments performed here have confirmed a reduction in the basal diffusion coefficient of the A316T receptor under basal conditions, indicative of a clustered phenotype. Additionally, the A316T variant might also be changing the capacity of the receptor to oligomerise with other receptors, such as the GIPR, which could influence its internalisation and clustering behaviour (605), a possibility that has not been examined here.

Effect of the A316T variant on G protein and β -arrestin 2 recruitment

Previous studies have shown a GoF mini-Gs recruitment profile for the A316T variant in HEK293T cells and a modest reduction in β -arrestin 2 recruitment (518, 601). NanoBiT complementation assays performed here to determine the coupling of each receptor with specific signalling mediators including $G\alpha_s$, $G\alpha_i$ and β -arrestin 2, both in the presence and absence of agonists showed that the ligand-free A316T GLP-1R exhibited significant increases in mini-Gs coupling, with no change in β -arrestin 2 recruitment in INS-1 832/3 β -cells. This is another indication that GLP-1R internalisation can occur independently of β -arrestin 2 recruitment, as has been shown in several studies (362, 363). Interestingly, recruitment of mini- $G_{i/o}$ was also increased in the A316T expressing cells compared to WT controls. $G\alpha_{i/o}$ proteins are typically involved in GLP-1R desensitisation by inhibiting adenylate cyclase, reducing cAMP levels, and contributing to receptor internalisation (356). The increased mini- $G_{i/o}$ recruitment in the A316T variant might reflect a compensatory feedback mechanism to counterbalance the heightened basal activity and signalling through the $G\alpha_s$ pathway, thus regulating the receptor activity and preventing over-stimulation. An alternative explanation

could be that the variant receptor is influenced by a mechanism known as “G protein priming”, where initial coupling of one type of G protein, subsequently primes the receptor for the recruitment and activation of another type of G protein (606). For instance, the initial coupling of the A316T GLP-1R to $G\alpha_s$ proteins could induce conformational changes in the receptor, making it more favourable for subsequent $G\alpha_{i/o}$ protein recruitment. This sequential activation can enhance receptor signalling complexity and diversify the downstream effects.

Under agonist stimulation, the A316T variant mini- G_s and β -arrestin 2 recruitment profiles resembled those of the WT receptor, though with slightly more pronounced responses. Moreover, β -arrestin 2 coupling efficiency showed a non-significant trend towards increase with exendin-D3 and a decrease with exendin-F1. This resulted in a receptor bias between β -arrestin 2 and mini- G_s responses which indicated that the A316T receptor favours $G\alpha_s$ coupling with exendin-F1 and β -arrestin 2 recruitment with exendin-D3 relative to the WT receptor. This suggests that specific agonists can modulate signalling pathways differently in the presence of GLP-1R mutations. Overall, the findings of this thesis emphasise that the A316T mutation stabilises the receptor in a partially active state, leading to heightened basal activity, which could have important implications for receptor desensitisation and overall downstream signalling. Importantly, these results also highlight the complexity of GLP-1R signal transduction in the context of genetic variations like the A316T mutation, as some of these changes appear to be agonist-dependent.

Since the A316T receptor exhibits increased basal endocytosis and trafficking whilst maintaining robust $G\alpha_s$ coupling and signalling, I also investigated spatiotemporal signalling patterns such as signalling from endosomal compartments, which has previously been seen as altered before for this variant receptor (364). Using a bystander NanoBiT signalling assay with

an activated $G\alpha_s$ -recognizing nanobody (Nb37), I found no significant differences in the recruitment of Nb37 to the plasma membrane and endosomes between the ligand-free A316T and WT GLP-1R. There was only a non-significant increase in the ratio of endosomal to plasma membrane activity for the A316T variant, suggesting that its GoF phenotype is not solely due to increased endosomal $G\alpha_s$ activity. Instead, the A316T variant demonstrates nuanced changes in localised $G\alpha_s$ activation patterns, with differential responses to various agonists highlighting the complexity of its signalling dynamics. For example, the A316T GLP-1R had significantly improved endosomal activity in response to GLP-1 but decreased endosomal signalling with exendin-4. This increase in GLP-1-induced endosomal activity efficiency aligns with the increased internalisation profile observed for the A316T receptor when stimulated with GLP-1, supporting a model where specific agonists can differentially modulate spatiotemporal receptor signalling based on their interaction with different receptor variants, and again highlighting that the GLP-1 seems to have a separate interaction with the A316T GLP-1R compared to exendin-4-based agonists.

Effect of the A316T variant on calcium mobilisation and cAMP production

To comprehensively understand the downstream effects of the A316T mutation and obtain a broader view of the functional consequences of the variant beyond G protein and β -arrestin 2 coupling, I assessed key second messenger systems, cAMP and intracellular calcium, which are critical for insulin secretion and β -cell function, revealing subtle differences in agonist-induced acute downstream signalling responses, including an increase in the cAMP production in response to GLP-1. Conversely, no changes were seen for exendin-4, exendin-F1, exendin-D3 and semaglutide. These results align with previous studies showing lower or unchanged mini- G_s coupling in response to exendin-4 and semaglutide in HEK293 cells (518). Utilising time-

lapse FRET microscopy with a ^TEpac^{VV}-based cAMP biosensor targeted to endosomal compartments, I also quantified endosomal cAMP production. This analysis demonstrated a non-significant increase in endosomal cAMP activity in response to GLP-1, complementation assays, but no significant change in response to exendin-4 stimulation, results which are similar to the ones found with the cADDIS system.

In parallel to this, I used the calcium dye Cal-520AM and time-lapse fluorescence microscopy to assess intracellular calcium dynamics in response to various GLP-1R agonists and the antagonist exendin-9. Both receptor types displayed similar calcium responses to all agonists tested at different glucose levels (6 mM and 11mM). However, I noticed a tendency for increased calcium rises in response to the antagonist exendin-9 the A316T expressing cells. The similarity in calcium responses to the GLP-1R agonists between mutant and WT receptors suggests that the variant maintains a comparable agonist-induced signalling profile. Yet, the significant increase in calcium mobilisation in response to the antagonist highlights a unique aspect of the A316T signalling behaviour, indicating a higher level of activity even in the presence of an antagonist.

In conclusion, these findings highlight complex subtle signalling effects of the A316T variant in pancreatic β -cells. The distinct agonist-specific signalling profiles indicates distinct receptor conformations induced by different agonists in combination with the variant induced conformations, further altering signalling efficacy.

Future studies should employ high-throughput screening techniques to investigate calcium mobilisation and cAMP dynamics using various doses of GLP-1R agonists. Advanced fluorescence-based assays could be used to measure intracellular signalling responses across a range of different agonist concentrations. Microplate readers and automated imaging platforms

reduce error from confocal microscopy imaging related issues, enabling the simultaneous analysis of multiple samples, providing detailed kinetic profiles and dose response curves. This approach will offer comprehensive insights into the dose-dependent effects of different GLP-1R agonists on calcium and cAMP signalling pathways in the presence of the A316T variant. Also, given that $G\alpha_q$ recruitment also plays a distinct role in GLP-1R calcium mobilisation (607, 608), NanoBiT complementation assays should be used to assess this in the context of the A316T variant.

Effect of the A316T variant on receptor-protein interactions

To further understand the mechanisms controlling the actions of the A316T GLP-1R, AP-MS was used to identify potential proteins that interact with the variant receptor both in the basal and agonist-bound form. A recent study by our group used the same technique to identify over 80 crucial protein interactors which change their association with GLP-1R upon agonist stimulation, including novel interactors VAPB and SPHKAP, which play significant roles at ER MCSs (540).

Given the A316T variant increased basal trafficking and signalling profile, I expected enhanced interactions with these proteins in the ligand-free state. Indeed, the A316T variant exhibited markedly increased interactions with all previously identified proteins associated with agonist-activated GLP-1R, indicating an intrinsic GoF phenotype (540). Notable interactors included $G\alpha_s$, AP2, and Rab5 validating my NanoBiT findings of increased $G\alpha_s$ association. Interestingly, the A316T interaction with SUMO1, a protein associated with receptor SUMOylation, was now increased rather than decreased upon agonist stimulation for the A316T variant receptor, indicating a different control of receptor post-translational modifications elicited by the presence of the A316T mutation.

I identified an additional 22 GLP-1R interactors with decreased association to the agonist-activated A316T GLP-1R, contrary to the WT receptor. These included SPHKAP and VAPB, integral to ER-mitochondrial tethering and β -cell function. Functional annotation clustering revealed enriched agonist-induced associations for phospholipid metabolism and $G\alpha_s$ signalling pathways in the A316T variant, suggesting a shift towards lipid-related metabolic processes and G protein signalling. Conversely, interactors with reduced association upon GLP-1R A316T activation were linked to fatty acid degradation and metabolism pathways. The precise mechanisms by which interactors associated with these processes regulate GLP-1R action in pancreatic β -cells is still unclear but could be an interesting avenue for further research.

Further studies should focus on validating these altered interactions and investigating their functional consequences to understand the full impact of the variant on β -cell physiology. However, prior to conducting studies on the A316T GLP-1R, the precise mechanisms by which these important candidate interactors regulate WT GLP-1R function as well as the link between the receptor and the phospholipid biosynthesis and fatty acid degradation pathways should be characterised to enable an accurate comparison with the variant receptor.

4.3.2 Influence of the A316T variant on GLP-1R function in human EndoC- β H3

I have also used human pancreatic β -cells as a highly physiological model to assess the effect of the A316T variant. Although employing human receptors ensures that the experimental findings are applicable to human physiology, using a human pancreatic β -cell context is superior to using rodent cells as they are more physiologically relevant for studying human β -cell function. Moreover, pharmacological responses to GLP-1RAs are more likely to reflect

the effects of human patients, facilitating the development of effective and safe drugs. Firstly, I verified that the impaired cell surface expression phenotype of the A316T GLP-1R persists in human pancreatic β -cells. This consistent phenotype across different cell lines suggests inherent defective surface expression as a result of increased basal trafficking and turnover rate of the A316T GLP-1R.

Using the EndoC- β H3 model allows us to evaluate functional consequences, such as GSIS effects of the A316T GLP-1R variant. Expression of the A316T variant modestly enhanced GSIS both in basal and exendin-4 stimulated conditions compared to the WT receptor, showing that this variant has a functional advantage in insulin secretion despite its impaired cell surface expression leading to enhanced insulintropic effects. Despite being better than WT, the exendin-4 induced GSIS response in A316T expressing cells is impaired compared to agonist-independent response. Perhaps this can be explained by counter regulatory feedback mechanisms preventing overstimulation of the variant receptor, or by system saturation so that the increased basal signalling does not allow for proportional increases in agonist-induced effects to the same level as those found in WT receptors. To further investigate these hypotheses *in vivo*, I have generated a knock-in mouse model of the A316T variant receptor, as described in the next chapter of this thesis.

Table 4. 1 Summary of key differences between WT and A316T GLP-1R from *in vitro* studies in both basal and induced state.

Parameter	A316T vs WT basal	A316T vs WT induced
Cell surface expression	A316T reduces cell surface expression	N/A
Lysosomal targeting	A316T enhances lysosomal targeting; rescued by lysosomal inhibition	N/A
Conformational response	No change in baseline	A316T exhibits enhanced conformational shifts and ECD opening in response to GLP-1
Calcium mobilization	A316T enhanced calcium response to the antagonist Exendin-9	Minimal change in response to agonists
cAMP production	N/A	Minimal change in response to agonists
β -arrestin-2 recruitment	Unchanged	A316T shifts Ex-D3 bias towards β arr2
Mini-Gs recruitment	A316T exhibits increased basal recruitment	A316T shifts Ex-F1 bias towards G α s
Lipid raft clustering	A316T enhances basal clustering	Reduced clustering in response to Exendin-4
Ubiquitination	Unchanged	Enhanced ubiquitination in response to Exendin-4
Interactome	A316T increases basal interactions with main GLP-1R interactors	Altered interaction patterns compared to WT induced including VAPB, VAPA and SUMO
Membrane localization in EndoC- β H3	A316T reduces membrane localization consistent with rodent cells	Increased
GSIS in EndoC- β H3	A316T enhances basal GSIS in response to 11mM glucose	A316T impairs insulin secretion in response to Ex-4

Chapter 5: Metabolic phenotype of knock-in

hGlp1r^{A316T/A316T} mice

5.1 Introduction

The *in silico* and *in vitro* studies indicate that exchange of the alanine into threonine at the 316 position of the human GLP-1R confers beneficial GoF characteristics, such as an increase in conformational arrangements associated with receptor activation in the ligand-free simulations, increased ECD opening and reduced TM6-TM7 distance, resulting in increased $G\alpha_s$ signalling under basal conditions. This along with previous studies showing increased $G\alpha_s$ signalling, cAMP and calcium responses in HEK cells expressing the A316T GLP-1R variant suggests that the mutation confers a reduced propensity for T2D due to its GoF phenotype. Here, I have studied the role of the A316T variant in glucose homeostasis in a whole-body context, by generating genetically engineered humanised GLP-1R mouse model with the A316T variant knocked-in using CRISPR/Cas9 technology, as has been done before for the GCGR (509), and subjecting both mutant and wild-type control littermates to *in vivo* metabolic studies to assess parameters associated with glycaemic control.

5.1.1 Generation of *hGlp1r*^{A316T/A316T} and *hGlp1r*^{-/-} transgenic mouse lines

To generate transgenic A316T knock in mice models, I used genetic engineering by sgRNA/CRISPR-Cas9 using C57BL/6 *hGlp1r* knock in mouse line (*hGlp1r*^{+/+}) generated at

Taconic Artemis GmbH (Cologne, Germany) as a starting point. A single guide RNA (sgRNA) targeting the region flanking the 316 position (5'- GCCCATTCTCTTTGCCATTG-3') was selected using RGEN tool (<http://www.rgenome.net/cas-designer/>) inputting a target sequence present in exon 9 of *hGlp1r* flanking the rs10305492 single nucleotide polymorphism. One-cell stage *hGlp1r*^{+/+} embryos were electroporated together with opti-MEM containing RNP (ribonucleoprotein) complexes of S.p. Cas9 Nuclease enzyme protein with sgRNA and the donor single-stranded oligo DNA nucleotide repair template (ssODN-HDR) using the NEPA21 electroporator (NEPA GENE Co. Ltd. Chiba, Japan) as described in section 2.4.2. The donor ssODNs used in this study are:

Donor ssODN	Sequence
Forward	5'- CATGAACTACTGGCTCATTATCCGGCTGCCCATTTCTCTTTACCATTGG GGTGAACCTTCCTCATCTTTGTTCTGGGTCATCTGCAT-3'
Reverse	3'- ATGCAGATGACCCGAACAAAGATGAGGAAGTTCACCCCAATGGTAA AGAGAATGGGCAGCCGGATAATGAGCCAGTAGTTCATG-5'

The ssODNs were designed using the Alt-R CRISPR HDR design tool (<https://eu.idtdna.com/pages/tools/alt-r-crispr-hdr-design-tool/>), using the same target sequence as for the sgRNA. The ssODN was designed as a donor template facilitating the replacement of the alanine coding sequence with threonine in TM5 by homology directed repair (HDR), and to ensure that the modified sequence is not targeted by the CRISPR-Cas9/sgRNA for cutting post-editing (609). Whilst previous HDR-directed repair methods to generate specific mutations used dsDNA plasmids as donor templates for the repair, ssODNs

circumvent the undesired random integration of the template into the genome, facilitating the successful introduction of defined point mutations. Moreover, optimised electroporation methods using sgRNAs, Cas9 protein and ssODNs are a more efficient method compared to microinjection of zygotes with dsDNA donors (609). Electroporation has also been shown to result in precise knock-in mutations in 25-100% of analysed animals, comparable to microinjection (610).

Electroporated two-cell stage embryos were placed into oviducts of pseudo-pregnant *hGlp1r*^{+/+} foster mothers. Upon analysis of animal genotypes by sequencing, using electroporation and an ssODN donor template resulted in precise and successful introduction of the A316T variant in 23% (three out of thirteen pups) of the mice. The rest of the mice I sequenced were either wild-type or had acquired indel deletions in regions preceding or after the mutation resulting in frameshift mutations that alter downstream protein coding, leading to a nonfunctional GLP-1R or a premature stop codon, and so a GLP-1R knockout. The observation of frameshift mutations amongst my animals could be explained by the repair pathways involved in the double strand break repair process (DSB). Post DSB by the CRISPR-Cas9/gRNA system, the cell can repair the DSB through two main pathways: non-homologous end joining (NHEJ) or homology-directed repair (HDR). When the intended outcome is to introduce a specific SNP precisely, CRISPR-Cas9 and HDR are used in conjunction with ssODNs. The ssODNs serve as a template for HDR, whereby the cell uses it to repair the DSB by incorporating the SNP precisely.

Subsequently, this method generated three parental mice (*hGlp1r*^{A316T +/+}) which were crossed to establish my transgenic line including the three genotypes *hGlp1r*^{+/+} (wild-type controls), *hGlp1r*^{A316T +/-} (heterozygous breeders used to maintain the colony), and *hGlp1r*^{A316T/A316T}.

These parent founders and future transgenic mice were identified and genotyped by performing qPCR analysis of genomic DNA from mice ear samples followed by purification of the qPCR product (534 base pairs) and Sanger sequencing.

5.2 Results

5.2.1 The A316T variant has no effect on body weight but reduces random-fed glucose under chow diet

To assess whether the introduction of the A316T mutation can affect body weight and blood glucose levels, the body weight and fed glucose (glucose levels post-glucose bolus) were monitored in a mixed-sex cohort of *hGlp1r*^{A316T/A316T} (A316T) and *hGlp1r*^{+/+} (WT) littermate controls at 12 weeks old under a normal chow diet. I assessed the physiological effects of the A316T mutation in 12-week-old mice which are considered as young adults, and by this point the β -cells are fully matured. My results show no significant difference in weight (Figure 5.1A, $p=0.6711$), but a significant reduction in fed-glucose levels in *hGlp1r*^{A316T/A316T} mice compared to *hGlp1r*^{+/+} littermate controls (Figure 5.1C, $p=0.045$). This indicates that mice belonging to both genotypes started the experiments with comparable body weights, ensuring that any differences in glucose metabolism are not due to differences in body weight.

5.2.2 The A316T variant enhances oral glucose tolerance and reduces fasting glucose under chow diet

To assess the glycaemic effects of the A316T mutation, oral glucose tolerance tests (OGTTs) involving oral administration of glucose mimicking the natural route of glucose intake in feeding were performed, as described in section 2.4.4, on *hGlp1r*^{A316T/A316T} and *hGlp1r*^{+/+} littermate controls under a normal chow diet. This specifically engages enteroendocrine gut cells, triggering the secretion of gut hormones including GLP-1, which is crucial for the enteroinsular axis (the coordinated response of the gut and pancreas to ingested nutrients) and thus enabling the evaluation of changes in the “incretin effect” and normal physiological pathways involving the endogenous GLP-1 as a result of the A316T mutation. The *hGlp1r*^{A316T/A316T} mice displayed significantly lower blood glucose levels at 10-, 30- and 60-minutes post-glucose bolus administration compared to their *hGlp1r*^{+/+} counterparts. The AUC analysis further confirms this difference, showing significantly lower AUCs in mice possessing the *hGlp1r*^{A316T/A316T} genotype compared to wild-type controls (Figure 5.1B, $p=0.0144$). This suggests that the A316T mutation in the human *Glp1r* enhances oral glucose tolerance and the incretin effect, indicating an ameliorated ability to manage glucose load efficiently. In humans, the 2-hour glucose test, part of the OGTT, is a critical diagnostic tool used to assess the body’s ability to utilise and clear glucose effectively. In my case, I assessed 1-hour glucose levels as part of the OGTT test, observing significantly lower 1-hour glucose levels in *hGlp1r*^{A316T/A316T} compared to *hGlp1r*^{+/+} control littermate controls, and thus suggesting more effective control of blood glucose levels (Figure 5.1D, $p=0.0286$). Interestingly, glucose levels were also significantly reduced post a 5-hour fast in these mutant

mice (Figure 5.1E, $p=0.0003$). This further supports the observation that the A316T mutation positively affects glucose homeostasis, leading to prolonged glucose euglycemia after glucose ingestion. Alternatively, the A316T variant may exert a direct effect on basal glucose homeostasis, independent of nutrient-stimulated GLP-1 secretion. This could involve enhanced constitutive ligand-independent signalling of the GLP-1R, resulting in reduced hepatic glucose production as a result of increased insulin and reduced glucagon secretion, as well as increased peripheral glucose uptake under fasting conditions. Such a mechanism is consistent with the observed reduction in fasting glucose levels and could contribute to the overall improvement in glycaemic control of *hGlp1r^{A316T/A316T}* mice.

5.2.3 The effects of the A316T variant on glucose responses in the absence and presence of GLP-1RAs

Basal glucose responses in hGlp1r^{A316T/A316T} mice

Next, I directly examined glucose metabolism under more controlled conditions using an intraperitoneal glucose tolerance test (IPGTT) as mentioned in section 2.4.5, investigating the pharmacodynamic and pharmacokinetic effects of GLP-1RAs in the presence of the A316T variant. IPGTTs also allow us to observe immediate physiological responses (0 hr i.e. immediately after treatment administration) as well as longer term responses (after 6 hours of treatment administration) to glucose (vehicle) $\pm$ GLP-1RA (2 nmol/kg exendin-4 or exendin-F1) treatment. Figure 5.1F illustrates the glucose levels over time in response to vehicle treatment with chow after 0 and 6 hours of treatment administration. IPGTT results showed that *hGlp1r^{A316T/A316T}* mice exhibited significantly decreased blood glucose levels at several time points after acute (0 hr) administration of the vehicle alone, with a similar less pronounced trend observed after six hours (6 hr), whereby the difference was significant only at the 10-

minute time point (Figure 5.1F). In a similar vein, comparing the AUC for glucose levels under the above conditions shows consistently lower AUC values for the *hGlp1r*^{A316T/A316T} mice relative to the *hGlp1r*^{+/+} wild type controls (Figure 5.1G), particularly noticeable after 0-hour intervention (p=0.0361), but not reaching statistical significance after the 6-hour intervention (p=0.1360).

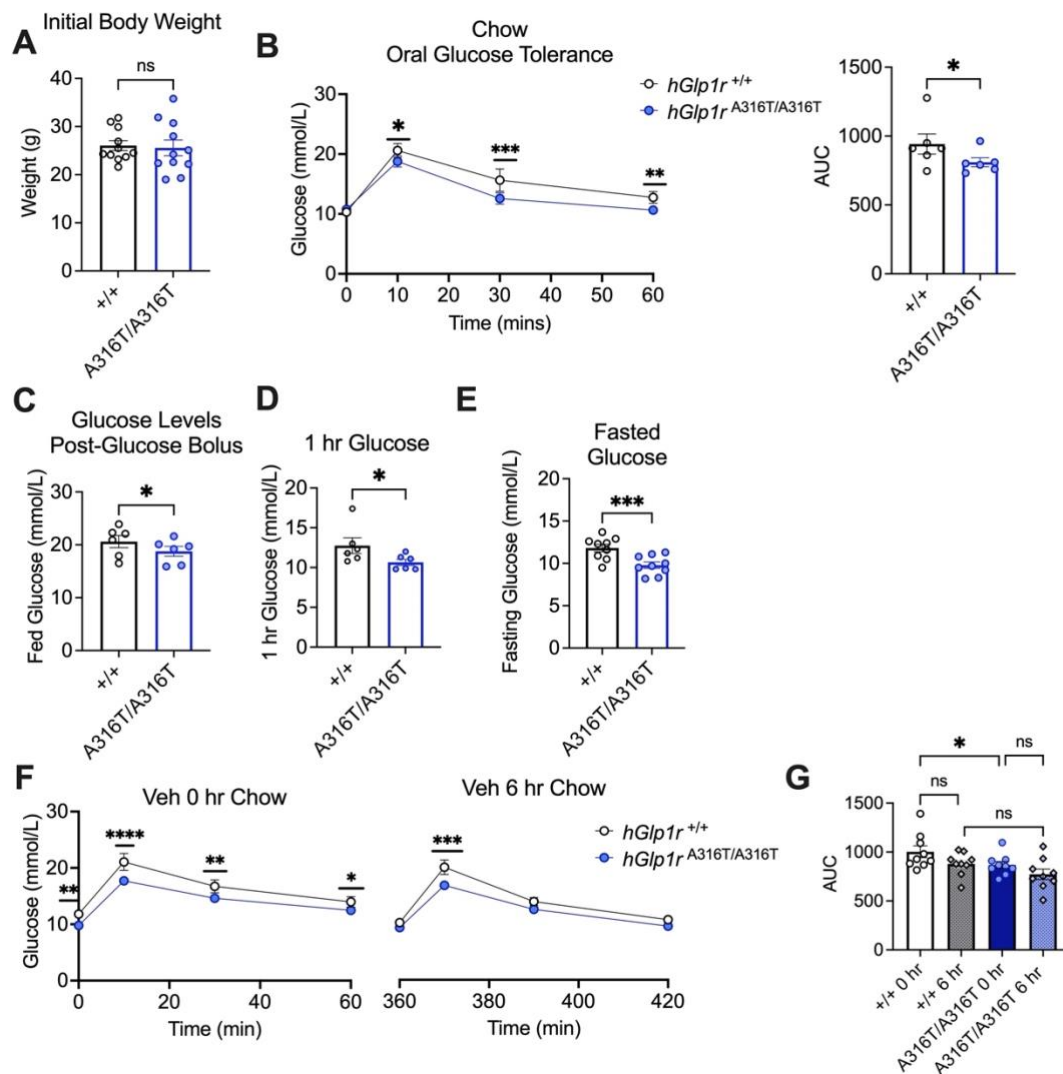


Figure 5.1 Impact of the A316T mutation on basal glucose tolerance and metabolic parameters in *hGlp1r*^{+/+} and *hGlp1r*^{A316T/A316T} mutant mice on chow diet. A) Initial body weight comparison between *hGlp1r*^{+/+} and *hGlp1r*^{A316T/A316T} mice. B) OGTT responses (2 g/kg of body weight glucose) and corresponding AUC following glucose administration into the gut via oral gavage after 5 hours of fasting in a mixed sex cohort of lean, adult

hGlp1r^{A316T/A316T} versus wildtype *hGlp1r^{+/+}* mice on chow diet (n = 6) C) Fed-glucose levels measured immediately post glucose bolus administration in the same mice. D) 1-hour glucose levels measured 1-hour post glucose bolus administration in the same mice. E) Fasted glucose levels (5 hours fast) from mice in (F). F and G) IPGTTs (2 g/kg of body weight glucose intraperitoneally) performed concurrently with (0 hr: acute), or 6 hours after (6 hr: long-term) intraperitoneal administration of vehicle (saline). Glucose curves (F) and corresponding AUC (G) shown; n=9 mice per genotype; age 16-18 weeks. Comparisons of A316T vs WT littermate controls were made with paired t-tests, one- or two-way analysis of variance (ANOVA) with Sidak's post-hoc tests. *p < 0.05, **p < 0.01, and ***p < 0.0001; ns=not significant. Data are presented as means ± SEM.

Agonist-induced glucose responses in hGlp1r^{A316T/A316T} mice

In terms of agonist-induced glucose responses, IPGTTs were performed following administration of the GLP-1RAs exendin-4 and exendin-F1 at a dose of 2 nmol/kg. Glucose curves illustrated in Figure 5.2A reveal that *hGlp1r^{A316T/A316T}* responses to exendin-4 resembled those of the *hGlp1r^{+/+}* at both 0 and 6 hours post-exendin-4 administration, with the *hGlp1r^{A316T/A316T}* mice exhibiting blunted 6-hour responses. Similarly, although administration of exendin-4 acutely (0 hr) significantly reduces glucose levels in both wild-type *hGlp1r^{+/+}* and *hGlp1r^{A316T/A316T}* mice, long term (6-hour) administration of exendin-4 resulted in glucoregulatory loss in both genotypes, associated with exendin-4-induced receptor desensitisation effects (Figure C, E, I and J). Notably, this glucoregulatory loss was more pronounced in *hGlp1r^{A316T/A316T}* mice in comparison to both vehicle responses alone and wild-type exendin-4 responses (Figure 5.2C and F). The ΔAUC, corresponding to agonist-minus vehicle-treated AUC differences, supports this observation, showing significantly higher ΔAUC values in *hGlp1r^{A316T/A316T}* mice compared to wild-type both acutely and at the 6-hour time-point, where exendin-4 treatment resulted in an apparent paradoxical increase in blood glucose levels compared to vehicle administration. These results highlight the impaired

glucose-lowering efficacy of exendin-4 in the mutant mice and the superiority of the inherent variant responses over agonist-induced responses (Figure 5.2F).

In parallel to the above results, a similar trend was observed for the biased agonist exendin-F1, also administered at a dose of 2 nmol/kg. Both mouse genotypes showed a significant reduction in blood glucose levels after acute agonist administration, with *hGlp1r^{A316T/A316T}* responses resembling those of the wild-type mice (figure 5.2B and D). Although acute responses were significantly reduced in *hGlp1r^{A316T/A316T}* mice, both wild-type and *hGlp1r^{A316T/A316T}* mice exhibited improved lower glucose levels compared to vehicle in response to exendin-F1 at 6-hour post-agonist administration, indicative of reduced receptor desensitisation of the biased agonist and superior prolonged responses *versus* exendin-4 of the A316T variant (Figure 5.2D and H). In fact, *hGlp1r^{A316T/A316T}* mice showed significantly lower glucose levels at 30 and 60-minutes post exendin-F1-administration compared to wild-type controls. The AUC and Δ AUC analysis in Figure 5.2G and H further confirmed that the prolonged glucose-lowering effect of exendin-F1 was maintained in *hGlp1r^{A316T/A316T}* mice (Figure 5.2J and H), resulting in the absence of the paradoxical glucose increases observed at the 6-hour time point for exendin-4.

I also calculated the standard metric “fold change over vehicle” to account for baseline differences, isolating the specific effect of the GLP-1RAs, and providing a clear picture of the efficacy of treatment between the two different genotypes. The fold change results, as shown in Figure 5.2K, indicate that exendin-4 and exendin-F1 are as effective at lowering glucose levels in *hGlp1r^{A316T/A316T}* mice as in wild-type controls administered acutely. Yet, folded AUC values were significantly higher post-Ex-4 administration for 6-hours post agonist administration in *hGlp1r^{A316T/A316T}* mice ($p=0.0009$), whilst no change was observed for

exendin-F1 relative to wild-type controls ($p=0.1501$), further confirming the loss of glucoregulatory response and receptor desensitisation to exendin-4 in these mutant mice. Comparing the difference between long-term (6 hour) and short-term (0 hour) agonist responses ($\Delta\Delta\text{AUCs}$) in *hGlp1r^{A316T/A316T}* mice (Figure 5.2L), revealed no significant differences in prolonger over acute effects for either agonist per genotype.

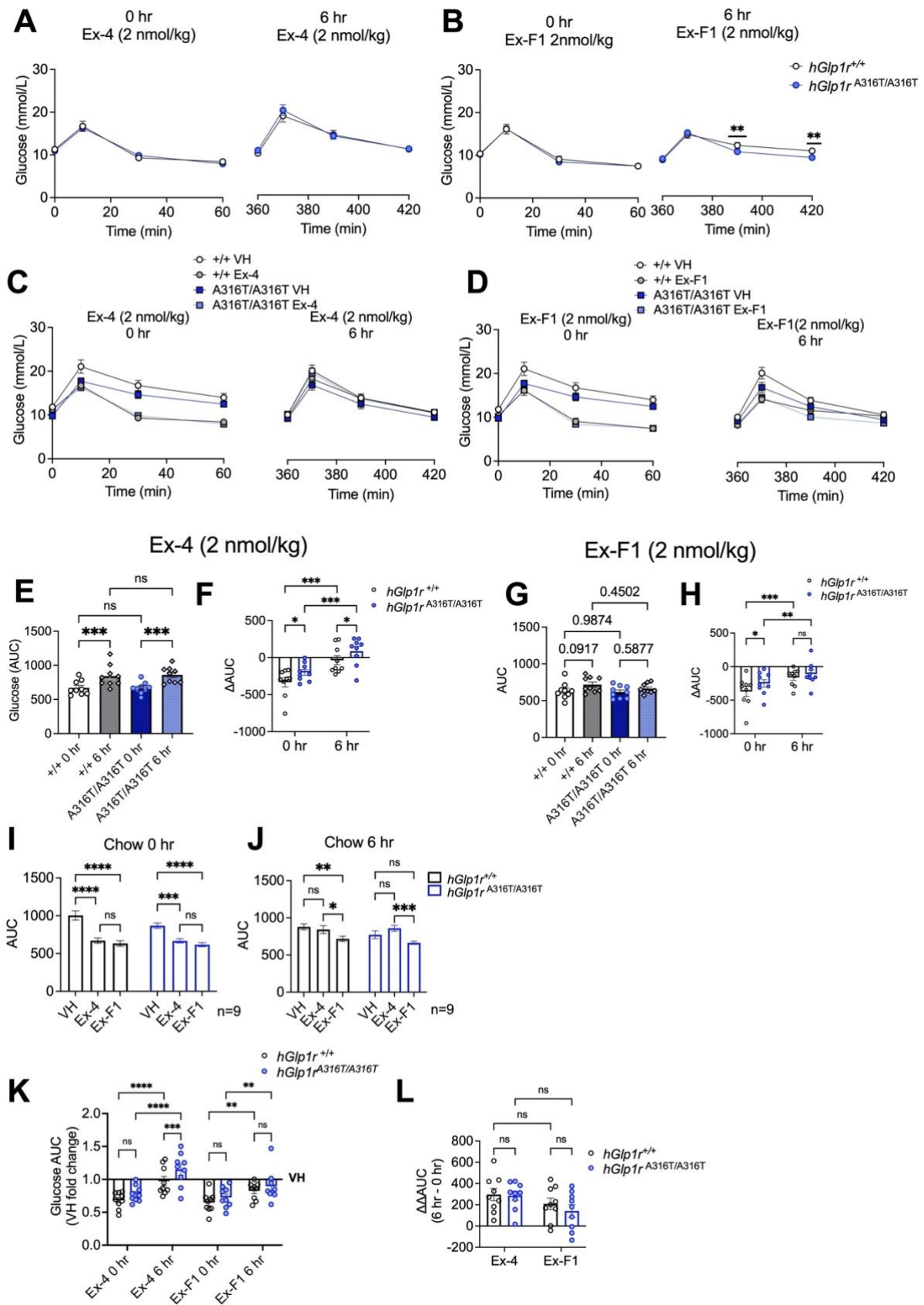


Figure 5.2 In-vivo agonist-induced GLP-1R responses in *hGlp1r*^{+/+} and *hGlp1r*^{A316T/A316T} mutant mice on chow diet. A-D) Glucose curves depicting results from IPGTTs (2 g/kg of body weight glucose intraperitoneally) performed concurrently with (0 hr: acute), or 6 hours after (6 hr: long-term) intraperitoneal administration 2 nmol/kg Exendin-4 (Ex-4) (**A and C**) or Exendin-F1 (Ex-F1) (**B and D**); n=9 mice per genotype; age 16-18 weeks. **E and G**) Corresponding AUCs calculated from glucose curves (A) and (B), respectively. **F and H**) Vehicle subtracted Δ AUC shown for data from E and G, respectively. **I and J**) AUC comparison for chow-fed mice under all treatments including vehicle (VH) at the 0 hr (**I**) and the 6 hr (**J**) time point. **K**) Fold AUCs for agonist-induced responses to Ex-4 and Ex-F1 at both the 0 hr and 6 hr time points, for data from I and J. **L**) $\Delta\Delta$ AUC values (6 hr-0 hr) from data in F and H. Comparisons of A316T vs WT littermate controls were made with one- or two-way analysis of variance (ANOVA) with Sidak's post-hoc tests. *p < 0.05, **p < 0.01, and ***p < 0.0001; ns=not significant. Data are presented as means $\pm$ SEM.

5.2.4 The A316T variant reduces weight gain and maintains enhanced oral glucose tolerance under a high fat, high sucrose diet

As GWAS studies showed that the A316T mutation is protective against T2D, I aimed to determine whether the effects of the mutation will be maintained under diet-induced metabolic stress conditions. To induce diet-induced metabolic stress, animals from section 5.2.3 including *hGlp1r*^{A316T/A316T} and wild-type littermate control mice were fed a high-fat high-sucrose diet (HFHSD, 58% kcal%) for 12-14 weeks.

Weight gain and body weight

After 14 weeks on the HFHSD, I observed that the *hGlp1r*^{A316T/A316T} mice showed markedly lower rate of weight gain compared to wild-type littermate controls over the course of the diet regimen, with the highest significant difference in weight exhibited between weeks six to eleven, indicating a protective effect from diet-induced obesity (Figure 5.3A). The associated AUC for weight gain also shows a decrease in the mutant mice (p=0.0505). Figure 5.3B confirms that there was no difference in final body weight of *hGlp1r*^{A316T/A316T} mice compared

to wild-type controls after the HFHSD regimen period, as by that point the mutant mice had caught up with the wild-type in terms of weight gain.

OGTTs

Oral glucose tolerance was then measured as before. Consistent with results observed in lean animals, oral glucose tolerance was significantly improved in *hGlp1r^{A316T/A316T}* mice, with a significant decrease in blood glucose levels at the 10- and 30-minute time-points (Figure 5.3C). The AUC for glucose levels during the OGTT was also significantly lower in the mutant mice ($p=0.0266$), indicating improved glucose tolerance and a superior ability to manage glucose loads despite metabolic stress and obesity.

Fasting, post-glucose bolus and 1-hour glucose levels

Figures 5.3D, E and F present the glucose levels post-glucose bolus administration, at 1-hour post-glucose administration and prior to bolus administration after a 5-hour fast, respectively. The *hGlp1r^{A316T/A316T}* mice had significantly reduced glucose levels both immediately after glucose administration and at the 1-hour mark compared to wild-type littermate controls (Figure 5.3D and E, $p=0.0454$ and $p=0.0410$). Yet, unlike under chow diet conditions, there was no significant difference in fasted glucose levels between the *hGlp1r^{A316T/A316T}* and *hGlp1r^{+/+}* mice (Figure 5.3F).

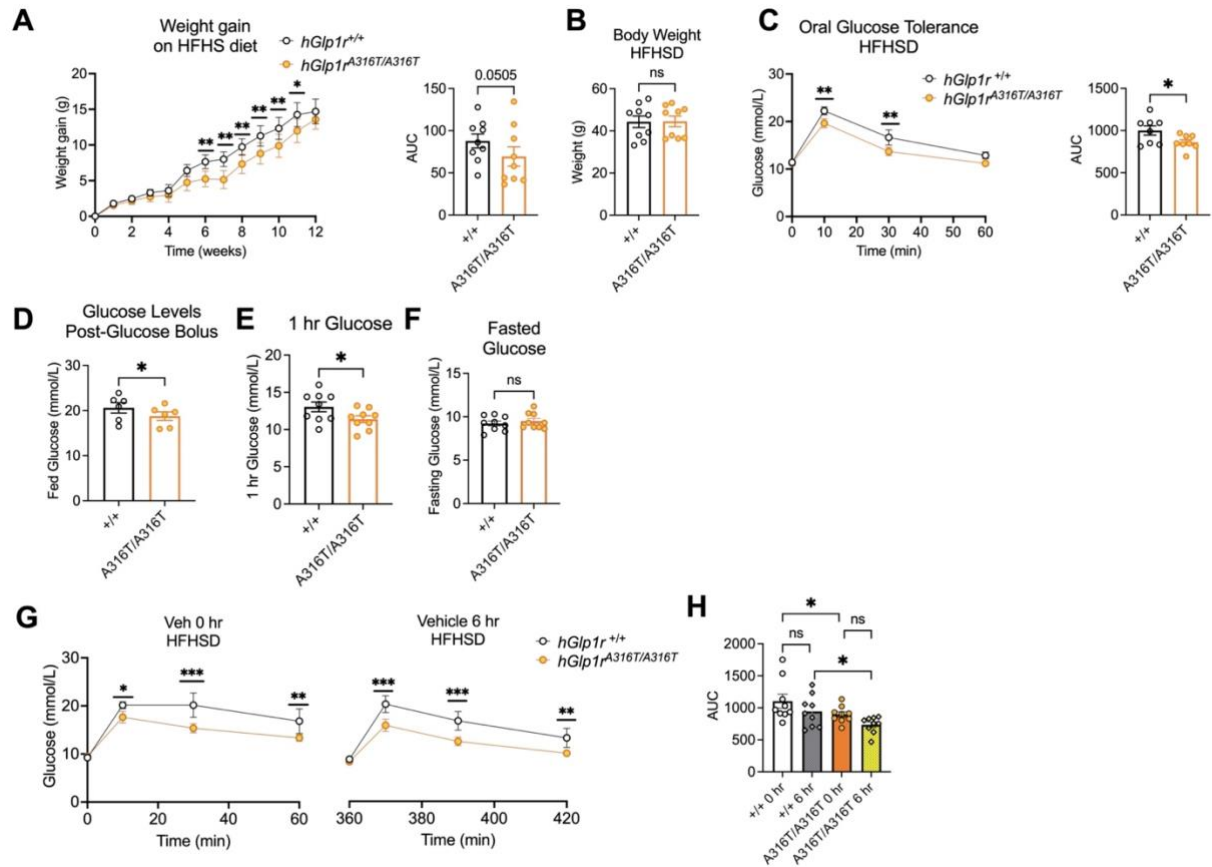


Figure 5.3 Impact of the A316T mutation on basal glucose tolerance and metabolic parameters in *hGlp1r*^{+/+} and *hGlp1r*^{A316T/A316T} mutant mice on HFHS diet. (A) Body weight gain curves of wild type (*hGlp1r*^{+/+}, black) and A316T (*hGlp1r*^{A316T/A316T}, yellow) mixed sex adult mice cohort from Figure 5.2 (n=9 for each genotype) maintained on a HFHS diet. (B) Body weight comparison between *hGlp1r*^{+/+} and *hGlp1r*^{A316T/A316T} mice after 12 weeks under HFHS diet. (C) OGTT responses (2 g/kg of body weight glucose) and corresponding AUC following glucose administration into the gut via oral gavage after 2 hours of fasting in a mixed sex cohort of lean, adult *hGlp1r*^{A316T/A316T} versus wildtype *hGlp1r*^{+/+} mice on chow diet (n = 9). (D) Fed-glucose levels measured immediately post glucose bolus administration from mice in (A). (E) 1-hour glucose levels measured 1-hour post glucose bolus administration from mice in (A). (F) Fasted glucose levels (5 hours fast) from mice in (A). (G and H) IPGTTs (2 g/kg of body weight glucose intraperitoneally) performed concurrently with (0 hr: acute), or 6 hours after (6 hr: long-term) intraperitoneal administration of vehicle (saline). Glucose curves (G) and corresponding AUC (H) shown; n=9 mice per genotype; age 16-18 weeks. Comparisons of A316T vs WT littermate controls were made with paired t-tests, one- or two-way analysis of variance (ANOVA) with Sidak's post-hoc tests. *p < 0.05, **p < 0.01, and ***p < 0.0001; ns=not significant. Data are presented as means ± SEM.

5.2.5 The effects of A316T variant on pharmacological GLP-1R responses in the absence and presence of GLP-1RAs under HFHSD

As in lean animals, to evaluate the effectivity of the A316T mutation in enhancing glucose levels in the presence and absence of GLP-1RAs under HFHSD-induced metabolic stress conditions, I performed IPGTTs as in section 5.2.3.

Basal glucose responses in $hGlp1r^{A316T/A316T}$ mice

The $hGlp1r^{A316T/A316T}$ mice consistently showed significantly lower glucose levels compared to $hGlp1r^{+/+}$ control mice, particularly notable at 10-, 30- and 60- minute time-points acutely and at these same time points but more pronounced at the 6-hour time point, consistent with findings observed in the lean animals. This trend indicates a persistent improvement in glucose regulation in the mutant mice. Figure 5.3H provides the comparative AUC values for glucose levels in Figure 5.3G. Here the data also shows significantly lower AUC values in $hGlp1r^{A316T/A316T}$ mice acutely ($p=0.0408$) and at the 6-hour time point ($p=0.0415$), highlighting the inherently positive impact of the A316T mutation on glucose metabolism.

Agonist-Induced Glucose Responses in HFHSD-fed $hGlp1r^{A316T/A316T}$ mice

Next, I wanted to investigate if the A316T associated agonist-induced glucose responses observed in lean animals remain when these animals are subjected to the HFHSD. Figures 5.4A and C illustrate the glucose responses to exendin-4 treatment at 2 nmol/kg. Both $hGlp1r^{A316T/A316T}$ and $hGlp1r^{+/+}$ mice experienced a reduction in glucose levels acutely at 0 hours post-treatment with exendin-4 (Figure 5.4C and I). However, the glucose-lowering effect was significantly less pronounced in the mutant mice (Figure 5.4C and F). Upon long-

term administration (6-hour time point), unlike under chow conditions, the exendin-4 agonist retained its ability to lower glucose in *hGlp1r^{+/+}* mice, but it was less effective in *hGlp1r^{A316T/A316T}* mice as highlighted by the lack of difference in vehicle- versus agonist-induced glucose AUCs (Figure 5.4C and J). Similar to exendin-4, exendin-F1 administration reduced glucose levels in both wild-type and mutant mice (Figure 5.4D and I) acutely, yet Δ AUC values revealed that as with exendin-4 this was significantly less pronounced in the *hGlp1r^{A316T/A316T}* compared to *hGlp1r^{+/+}* mice (Figure 5.4H, $p=0.009$). Surprisingly, prolonged 6-hour responses to exendin-F1 resembled those of exendin-4 in the control *hGlp1r^{+/+}* mice, with both agonists retaining the ability to lower glucose levels significantly compared to vehicle conditions (Figure 5.4J, $p=0.0021$). Yet, the opposite was seen in *hGlp1r^{A316T/A316T}* mice, whereby exendin-F1 treatment elicited no significant reduction in glucose levels compared to vehicle conditions (Figure 5.4B, D and J).

These effects were also apparent when I performed a comparative analysis of corrected glucose AUC values and fold-changes. The data emphasise significant differences in glucose responses between wild-type and mutant mice, with *hGlp1r^{A316T/A316T}* mice showing consistently higher Δ AUC and fold-change values for both exendin-4 and exendin-F1 treatments both acutely and in the long-term, signifying a reduced therapeutic effect of these agonists in the presence of the A316T mutation, and bolstering the superior effect of the variant independently of agonist treatment. Lastly, comparing the difference between long-term (6 hour) and short-term (0 hour) agonist responses ($\Delta\Delta$ AUCs) in *hGlp1r^{A316T/A316T}* mice (Figure 5.4L), revealed that long-term responses to both agonists studied are worse than acute responses in both genotypes, but the defect in long-term versus acute responses to exendin-4 was significantly more pronounced in *hGlp1r^{A316T/A316T}* mice (Figure 5.4L, $p=0.0223$). In

addition to this, whilst in lean animals the *hGlp1r*^{A316T/A316T} mice exhibited a modest decrease in the defect in long-term responses to exendin-F1 relative to wild-type controls, this was not the case in the HFHS-fed mice (Figure 5.2L and 5.4L).

Therefore, on a background of metabolic stress, long-term administration of both exendin-4 and exendin-F1 can no longer reduce glucose levels more than vehicle alone in *hGlp1r*^{A316T/A316T} mice.

Figure 5.4 *In-vivo* agonist-induced GLP-1R responses in *hGlp1r*^{+/+} and *hGlp1r*^{A316T/A316T} mutant mice on HFHS diet. **A-D)** Glucose curves depicting results from IPGTTs (2 g/kg of body weight glucose intraperitoneally) performed concurrently with (0 hr: acute), or 6 hours after (6 hr: long-term) intraperitoneal administration 2 nmol/kg Exendin-4 (Ex-4) (**A and C**) or Exendin-F1 (Ex-F1) (**B and D**); n=9 mice per genotype; age 28-30 weeks. **E and G)** Corresponding AUCs calculated from glucose curves (A) and (B), respectively. **F and H)** Vehicle subtracted Δ AUC shown for data from E and G, respectively. **I and J)** AUC comparison for HFHS diet fed mice under all treatments including vehicle (VH) at the 0 hr (**I**) and the 6 hr (**J**) time point. **K)** Fold AUCs for agonist-induced responses to Ex-4 and Ex-F1 at both the 0 hr and 6 hr time points, for data from I and J. **L)** $\Delta\Delta$ AUC values (6 hr-0 hr) from data in F and H. Comparisons of A316T vs WT littermate controls were made with one- or two-way analysis of variance (ANOVA) with Sidak's post-hoc tests. *p < 0.05, **p < 0.01, and ***p < 0.0001; ns=not significant. Data are presented as means $\pm$ SEM.

5.2.6 The effects of the A316T variant on pharmacological responses to long-acting clinical agonists

To interrogate whether these A316T associated effects would also be elicited by clinically leading agonists, I performed an analogous study in the same mice cohort of HFHSD-fed animals with the long-acting GLP-1R agonists semaglutide and the dual GLP-1/GIPR agonist tirzepatide (which shows reduced β -arrestin 2 recruitment at the GLP-1R but not at its preferentially binding GIPR) at 24 and 72 hours post-agonist injection.

*Basal 24 and 72-hour glucose responses in *hGlp1r*^{A316T/A316T} mice*

I observed a similar profile to previous experiments in section 5.2.5 for *hGlp1r*^{A316T/A316T} animals, with a significantly enhanced glucose tolerance 24 and 72 hours after vehicle administration compared to the *hGlp1r*^{+/+} mice, confirming that the mutation significantly improves glucose metabolism under vehicle conditions (Figure 5.5A and B). Notably, the most significant reductions in glucose levels occur at 10-, 30- and 60- minute time points for both acute and 6-hour IPGTTs. The AUC analysis in Figure 5.5B supports this, showing a

significant decrease in glucose levels in $hGlp1r^{A316T/A316T}$ mice both 24- and 72-hours post-vehicle treatment, with a more pronounced effect at 24 compared to 72 hours ($p = <0.0001$ and $p = 0.0035$).

Agonist-induced glucose responses at 24 and 72 hours in 24 and 72-hour $hGlp1r^{A316T/A316T}$ mice

For semaglutide-induced glucose excursions at both 24- and 72-hours, data revealed slightly lower glucose levels exhibited by $hGlp1r^{A316T/A316T}$ mice, particularly at the 30-minute time point at the 24-hour IPGTT, and the 10-, 30- and 60-minute time points for the 72-hour IPGTT (Figure 5.5C). On the other hand, tirzepatide-induced glucose excursions were similar for both 24- and 72-hours IPGTTs (Figure 5.5E). As predicted, direct comparison of AUCs showed that at 24-hours post-semaglutide or tirzepatide administration in $hGlp1r^{+/+}$ mice, glucose levels were significantly lower than those with corresponding vehicle administration, but there was no difference between semaglutide and tirzepatide responses (Figure 5.5G). However, in $hGlp1r^{A316T/A316T}$ mice, semaglutide or tirzepatide administration did not significantly alter glucose levels, and tirzepatide paradoxically increased glucose levels in comparison to vehicle administration alone (Figure 5.5H).

Further calculations of the vehicle fold changes and Δ AUCs, provide support of the above results, showing that responses to 24-hour semaglutide and tirzepatide are significantly worse in $hGlp1r^{A316T/A316T}$ relative to wild-type control mice, whereby no change was observed in glucose levels in response to semaglutide and a significant increase in glucose levels was observed in response to tirzepatide relative to vehicle responses (Figure 5.5K). Conversely, no difference was seen between $hGlp1r^{A316T/A316T}$ and wild-type mouse responses 72-hours post-semaglutide administration, but a significant increase in fold-change values was seen in

hGlp1r^{A316T/A316T} mice in response to tirzepatide treatment (Figure 5.5K). The lack of difference seen between the genotypes may be because of loss of wild-type responses to the agonists 72-hours post-agonist administration as a result of receptor desensitisation. In addition to this, contrary to previous findings, prolonged glucose lowering responses to tirzepatide *versus* semaglutide were only modestly enhanced in the wild-type controls. This may be explained by the inherent defect in endogenous receptor cell surface expression in this mouse line, or by differences in tirzepatide responses in human *versus* mouse models (611).

Comparing the $\Delta\Delta$ AUCs further showed that 72-hour responses to tirzepatide are only slightly better than those of semaglutide in wild-type mice, yet long-term administration of both agonists offered no change compared to acute responses in *hGlp1r^{A316T/A316T}* mice as these were initially worse relative to both their own vehicle conditions as well as in comparison to the wild-types. This difference is significant between the two genotypes for tirzepatide administration (Figure 5.5L). The results of the semaglutide and tirzepatide treatment studies highlight a significant impairment in agonist-mediated glucose regulation, whereby both semaglutide and tirzepatide, which are potent long lasting GLP-1R agonists, were less effective in lowering glucose levels in mutant mice at both 24- and 72-hours post-administration.

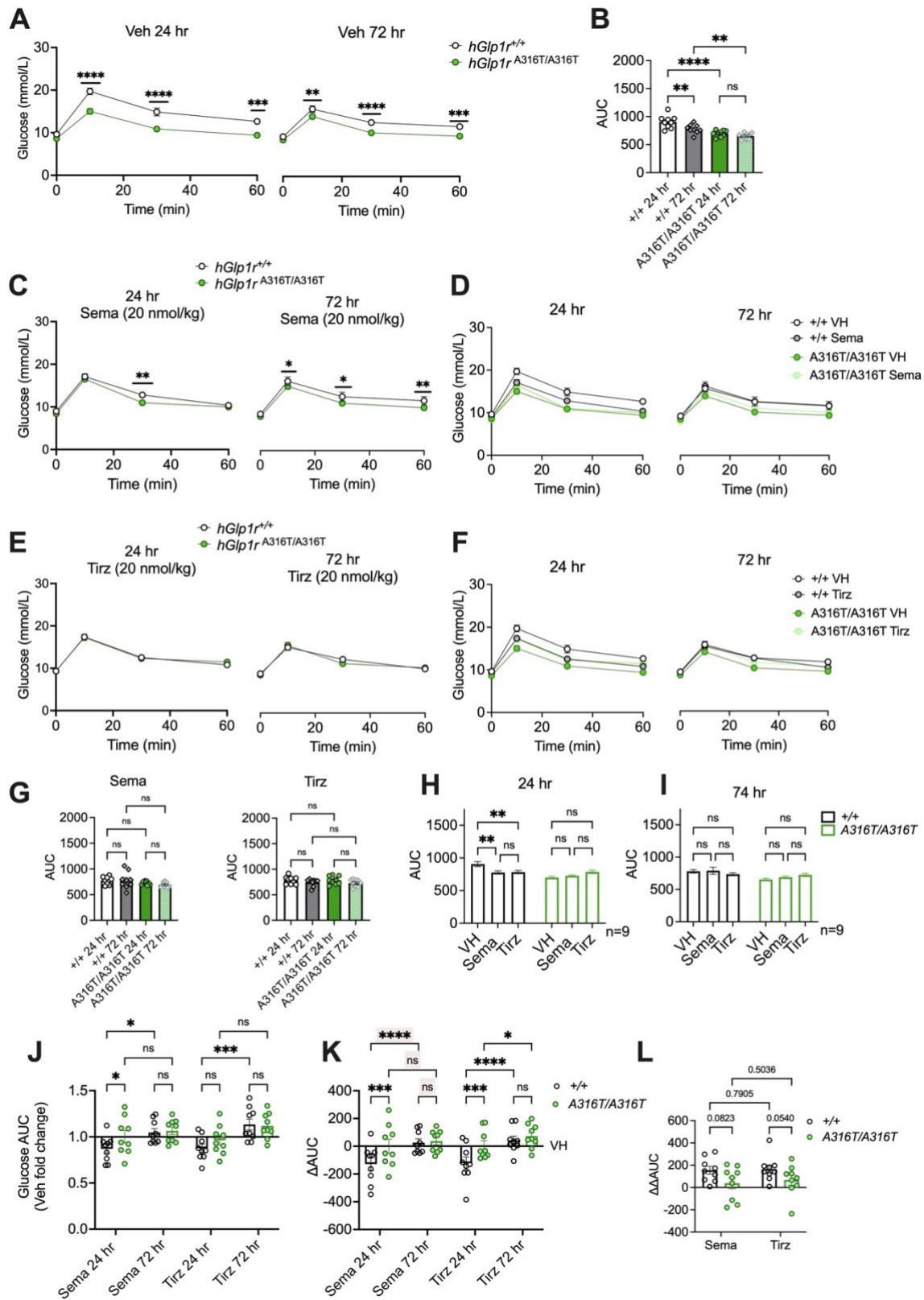


Figure 5.5 *In-vivo* long-acting clinical agonist-induced GLP-1R responses in *hGlp1r*^{+/+} and *hGlp1r*^{A316T/A316T} mutant mice on HFHS diet. Glucose curves depicting results from IPGTTs (2 g/kg of body weight glucose intraperitoneally) performed at 24 and 72 hours after agonist IP administration of vehicle (VH) (**A**), 20 nmol/kg Semaglutide (Sema) (**C and D**), or Tirzepatide (Tirz) (**E and F**) in the same mice cohort as Figure 5.4; n=9 mice per genotype; age 28-30 weeks. **B**) AUCs corresponding to data in glucose curves shown in A. **G and H**) AUC comparison for HFHS diet fed mice under all treatments including vehicle (VH) at the 24 hr (**G**) and the 72 hr (**H**) time point. **I and J**) Fold changes of AUCs (**I**) and vehicle subtracted AUCs (Δ AUC) for agonist-induced responses to Semaglutide and Tirzepatide at both the 24 hr and 72 hr time points, for data from G and H. **K**) $\Delta\Delta$ AUC values illustrating differences between 72 hr and 24 hr agonist responses from data in J. Comparisons of A316T vs WT littermate controls were made with one- or two-way analysis of variance (ANOVA) with Sidak's post-hoc tests. *p < 0.05, **p < 0.01, and ***p < 0.0001; ns=not significant. Data are presented as means $\pm$ SEM.

5.2.7 The A316T variant enhances insulin secretion *in vivo*

To test whether enhanced glucose tolerance observed as an A316T-associated phenotype was due to enhanced insulin secretion *in vivo*, plasma insulin was measured from blood samples taken at timepoint 10 minutes post-glucose $\pm$ agonist administration during the short-term and long-term IPGTTs. This time-point was chosen as it corresponds to the period at which the most significant differences between the two genotypes are observed.

In parallel to my IPGTT results, I observed that vehicle administration acutely (0 hours) elicited increased plasma insulin levels and thus insulin secretion in the *hGlp1r*^{A316T/A316T} compared to wild-type control mice, and this became significant for the 6-hour time-point (Figure 5.6A and B, p=0.0165). Whilst exendin-4 elicited significantly increased plasma insulin levels in wild-type mice both acutely and at the 6-hour time-point (p=0.0388 and p=0.0061), only a modest non-significant increase was observed for *hGlp1r*^{A316T/A316T} mice acutely (0 hours) (p=0.1382) and a slight decrease over a longer period (6 hours). Further vehicle fold-change calculations provide support for my IPGTT findings in Figure 5.6C, which shows reduced fold-changes over vehicle responses to acute exendin-4 administration in

hGlp1r^{A316T/A316T} compared to wild-type mice, and this became significant upon administration of exendin-4 over a longer 6-hour period, indicating an impaired ability for this agonist to promote insulin secretion in these mutant mice (Figure 5.6C, $p = 0.0347$). In parallel to this, I investigated plasma insulin levels under HFHSD-induced obesity to validate if this enhancement in insulin secretion is also maintained under metabolic stress. The data depicted in Figure 5.6D, confirms that plasma insulin levels are significantly enhanced in *hGlp1r^{A316T/A316T}* mice compared to wild-type controls independently of any agonist, consistent with findings observed in lean animals ($p = 0.0225$).

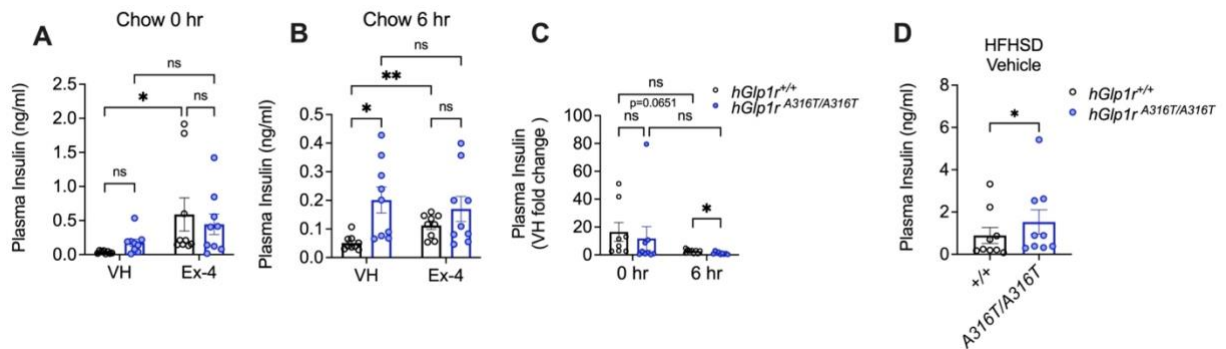


Figure 5.6 The A316T variant enhances *in vivo* plasma insulin levels in the absence of agonists under both chow and HFHS diet conditions. **A and B)** Plasma insulin levels from *hGlp1r^{+/+}* versus *hGlp1r^{A316T/A316T}* mutant mice on a normal chow diet collected during the 0 hr (**A**) and 6 hr (**B**) IPGTTs in response to vehicle (VH) and exendin-4 (Ex-4) treatment. **C)** Plasma insulin fold changes (normalized to VH), comparing agonist-induced responses in the two mice genotypes at both time points calculated from values in A and B. **D)** Plasma insulin levels from *hGlp1r^{+/+}* versus *hGlp1r^{A316T/A316T}* mutant mice on a metabolic stress inducing HFHS diet collected during the 0 hr IPGTT in response to vehicle (VH). Comparisons of A316T vs WT littermate controls were made with paired t-tests or two-way analysis of variance (ANOVA) with Sidak's post-hoc tests. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.0001$; ns=not significant. Data are presented as means $\pm$ SEM.

5.2.8 The A316T variant alters islet cytoarchitecture and α : β -cell ratios

The cellular composition and spatial arrangement of pancreatic islet cells are fundamental to maintaining glucose homeostasis. As mentioned previously in the introduction, the islets of Langerhans are primarily composed of glucagon-secreting α -cells and insulin-secreting β -cells, with other endocrine populations such as δ -, PP-, and ϵ -cells present in smaller proportions (612-615). In murine islets, β cells typically occupy the islet core, surrounded by a mantle of α - and other non- β endocrine cell; an organisation thought to facilitate paracrine signalling within the islet (614-616). This spatial arrangement is crucial for the regulation of insulin and glucagon secretion, although it can be altered under metabolic stress or in disease states. Notably, human islets exhibit a more intermingled distribution of α - and β -cells, indicating that islet cytoarchitecture is species-specific and functionally adaptable (617).

Alterations in the relative mass or spatial arrangement of α - and β -cells can lead to impaired insulin or glucagon secretion, contributing to diabetes. In fact, previous literature observes alterations in α - and β -cell mass that are central to the development and progression of T2D, including a reduction in β -cell mass due to increased apoptosis and de-differentiation, as well as α -cell hyperplasia resulting in elevated glucagon levels (618, 619).

On the other hand, GLP-1RAs have been shown to promote physiological processes that increase β -cell mass including increased β -cell proliferation through activation of pathways such as the cAMP/PKA pathway as well as reducing β -cell apoptosis by activating anti-apoptotic pathways reducing oxidative and ER stress (231, 232). However, the impact on α -cell mass is less clear. Some studies suggest that prolonged inhibition of glucagon secretion

by GLP-1R agonists could potentially lead to changes in α -cell mass, but this effect is not as well-characterised as the effects on β -cells (620). Examining the effect that the A316T mutation might have on islet cell composition could provide insights into underlying mechanisms by which the GLP-1R regulates glucose tolerance and metabolism. Changes in α - or β -cell mass can indicate a compensatory response to insulin resistance or an adaptive mechanism to metabolic stress. Additionally, alterations in the intra-islet localisation of α -cells can affect cell-cell communication within the islet, potentially disrupting the regulatory feedback mechanisms essential for maintaining glucose homeostasis.

Given that the A316T variant confers a GoF phenotype in glucose tolerance and plasma insulin levels independent of agonist stimulation, I hypothesised that these effects might lead to changes in islet cyto-architecture and β -cell identity in the *hGlp1r* knock-in mouse model. I therefore investigated changes in α - and β -cell mass in *hGlp1r^{A316T/A316T}* mice compared to wild-type controls under normal (chow) and metabolic stress conditions.

To do this I performed histological analysis of pancreatic tissue from both chow- and HFHSD-fed *hGlp1r^{A316T/A316T}* and *hGlp1r^{+/+}* mice. Immunofluorescence staining for insulin and glucagon was used to visualise β - and α -cells, respectively, with DAPI marking the nuclei. Glucagon staining was especially useful to confirm islet boundaries during quantification, as it allowed clear discrimination of true islets from insulin-positive regions with background signal. While insulin sometimes displayed low background signal outside islets, the combined presence of glucagon-positive α -cells and characteristic islet morphology provided confidence in that only true islets were analysed.

Immunolabelling of whole pancreatic sections from chow-fed *hGlp1r^{A316T/A316T}* mice revealed no significant differences in islet diameter, β -cell mass, α -cell mass, or α -to- β -cell ratio relative to wild-type *hGlp1r^{+/+}* mice (Figure 5.7A-D). This suggests that under chow diet conditions, the A316T mutation in the GLP-1R does not markedly alter α - or β -cell mass in the pancreas.

Next, I assessed if intra-islet architecture would remain unaffected by the A316T mutation under metabolic stress conditions of HFHSD. Figures 5.7E and F-I depict the changes in α - and β -cell mass in response to HFHSD. As with chow-fed animals, there was no significant change in the insulin-positive β -cell area in *hGlp1r^{A316T/A316T}* compared to wild-type controls (Figure 5.7G). Yet, remarkably I observed a significant increase in islet diameter, glucagon-positive α -cell area and α -to- β -cell ratio in these mutant mice ($p = 0.032$, and $p = 0.0049$), suggesting a disproportionate expansion of α -cells relative to β -cells under dietary stress (Figure 5.7F, H and I). Furthermore, I also observed a change in intra-islet localisation of α -cells in the *hGlp1r^{A316T/A316T}* mouse pancreas, with a significant proportion of α -cells losing peripheral localisation, typical of murine islet architecture, and gaining a core localisation resembling that characteristic of human islets (Figure 5.7I). On closer inspection of *hGlp1r^{A316T/A316T}* mouse islet intra-islet cyto-architecture, I observed a notable proportion of non-peripheral cells expressing both insulin and glucagon, potentially indicating a compensatory α -to- β -cell trans-differentiation effect (Figure 5.7E). This aberrant intra-islet localisation of α -cells observed under HFHSD, indicates a potential disruption in islet architecture triggered by metabolic stress with the A316T mutation.

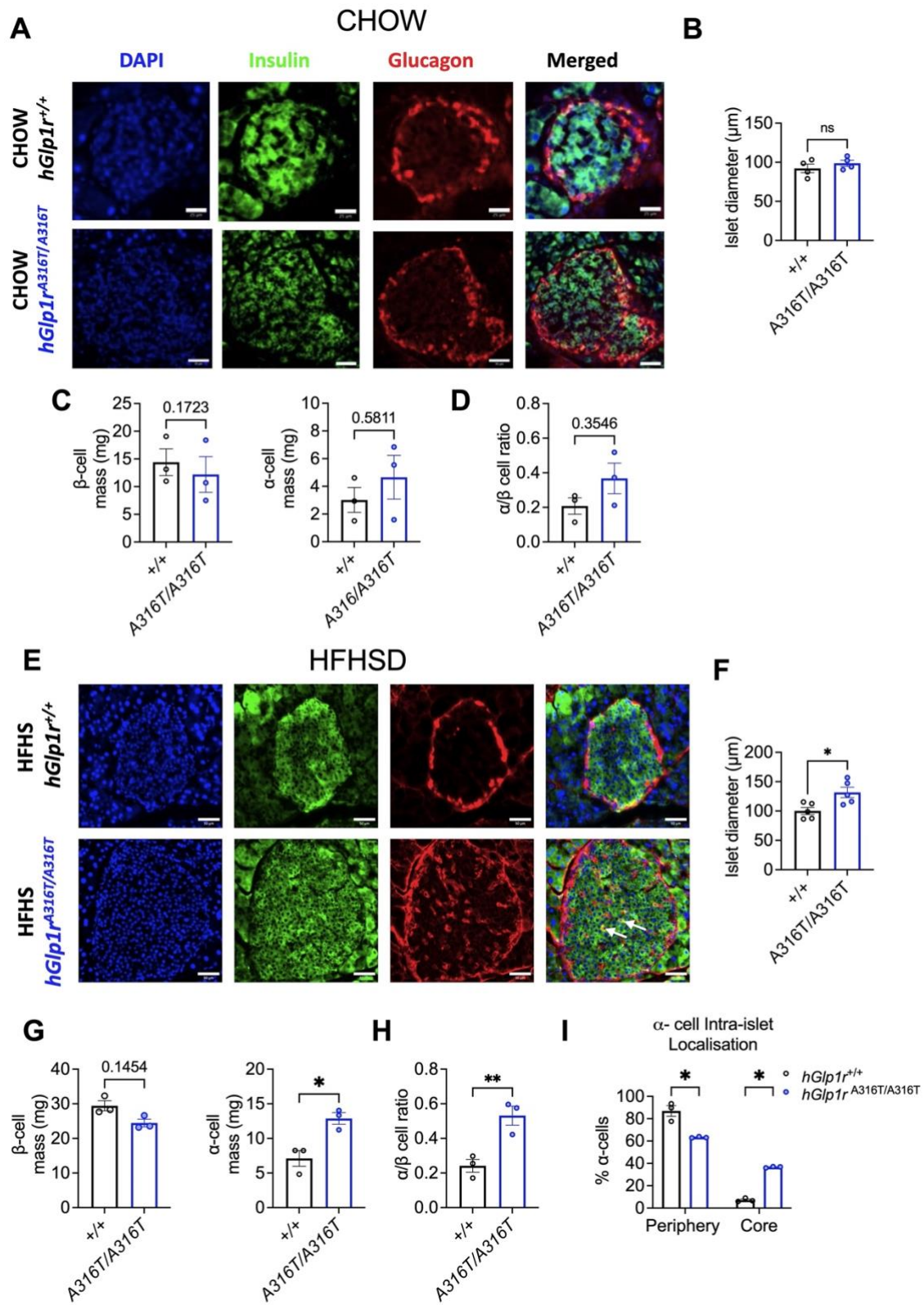


Figure 5.7 The *hGlp1r*^{A316T/A316T} mice on HFHSD show altered α -cell mass and intra-islet localization. Representative images showing immunofluorescence staining of pancreatic islets within frozen tissue sections isolated from chow- (A) and HFHS- (E) diet-fed *hGlp1r*^{+/+} control and *hGlp1r*^{A316T/A316T} mutant mice. DAPI (blue) marks nuclei, insulin (green) marks β -cells, and glucagon (red) marks α -cells. The merged images show islet structure and cell distribution. Scale bars represent 50 μ m. Quantification of islet diameter, β -cell mass, α -cell mass and α/β -cell ratio for both chow-fed (B-D) and HFHS-fed (F-H) mice. (I) Comparison of % α -cells in peripheral vs core islet localisation. Fifteen to twenty-five islets were counted per mouse. Comparisons of A316T vs WT controls were made with paired t-tests or two-way analysis of variance (ANOVA) with Sidak's post-hoc tests. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.0001$; ns=not significant. Data are presented as means $\pm$ SEM.

5.2.9 The A316T variant modestly affects gene expression associated with β -cell identity

Having observed marked differences in islet cyto-architecture and cell composition between the *hGlp1r*^{A316T/A316T} and the *hGlp1r*^{+/+} genotype, I sought to further understand the underlying mechanism behind these differences. To achieve this, I examined both enriched and disallowed gene expression in the context of these two genotypes. Enriched genes are those that are highly expressed in mature differentiated β -cells that allow them to carry out their function normally. Conversely, disallowed genes are housekeeping genes which are typically expressed at very low expression levels in mature β -cells, preventing them from interfering with specialised β -cells function and cellular identity. Thus, aberrant downregulation of enriched genes or upregulation of disallowed genes, can signal a loss of β -cell identity. By assessing the expression of these genes in islets isolated from *hGlp1r*^{A316T/A316T} and wild-type controls, I aimed to elucidate underlying molecular mechanisms by which the A316T mutation impacts islet cell identity, function, and mass. Moreover, changes in the expression of these

genes could provide important insights into the novel molecular mechanisms by which altered GLP-1R activity results in changes in islet cell identity, composition and function.

In this study, I investigated the “enriched” and “disallowed” gene expression profile of pancreatic islets isolated from the *hGlp1r^{A316T/A316T}* and *hGlp1r^{+/+}* wild type animals under two dietary conditions: standard chow and HFHSD.

Gene expression under chow diet

Figure 5.8A depicts the gene expression profiles of islets from mice maintained on a standard chow diet. The results showed the following patterns:

No significant difference was observed in gene expression of the β -cell “disallowed genes” *Ldha* (lactate dehydrogenase A), or *Slc16a1* (monocarboxylate transporter 1) between *hGlp1r^{A316T/A316T}* and *hGlp1r^{+/+}* islets, yet I observed a significant increase in *Acot7* (acyl-CoA thioesterase 7) in *hGlp1r^{A316T/A316T}* islets compared to wild-type controls (p= 0.0454). Additionally, there was a tendency for increased α -cell specific *Gcg* (glucagon) expression, indicating that glucagon gene expression is only mildly affected by the mutation under chow-fed conditions.

On the other hand, for β -cell “enriched genes” showed a tendency for upregulation, including *Ins1* (insulin), in *hGlp1r^{A316T/A316T}* versus *hGlp1r^{+/+}* islets. Nonetheless, *hGlp1r^{A316T/A316T}* islets exhibited significantly higher *Mafa* (Maf transcription factor A) expression compared to *hGlp1r^{+/+}* islets (p=0.0005). MAFA is critical for β -cell function and insulin gene expression, so that increased *Mafa* levels could contribute to improved β -cell function in A316T islets. Akin to this, I observed a non-significant increase in *Kcnj11* (potassium inwardly rectifying

channel, subfamily J, member 11) expression in *hGlp1r^{A316T/A316T}* islets (p= 0.0773). *Kcnj11* is involved in insulin secretion, and its increased expression could enhance this process in the presence of the A316T mutation.

Gene expression under HFHSD

Figure 5.8B illustrates the gene expression profiles of islets from animals belonging to the same genotypes as above but subjected to a HFHSD. The observed trends were as follows:

No significant difference was observed in gene expression of the β -cell “disallowed genes” *Ldha*, *Slc16a1* and *Acot7* between *hGlp1r^{A316T/A316T}* and *hGlp1r^{+/+}* islets, contrary to the significantly increased *Acot7* expression observed in islets under chow conditions. Additionally, the *Gcg* expression profile resembled that observed in chow-fed *hGlp1r^{+/+}* mouse islets, indicating that glucagon gene expression remains unaffected by the mutation under HFHSD-fed conditions.

Separately, similar to chow diet I observed a non-significant increase in *Kcnj11* expression (p=0.1379), as well as a non-significant increase in *Ins1* expression in *hGlp1r^{A316T/A316T}* compared to *hGlp1r^{+/+}* derived islets. In addition to this, *hGlp1r^{A316T/A316T}* islets also exhibited significantly higher expression of *Mafa*, consistent with chow-diet findings. The analysis of enriched and disallowed gene expression in *hGlp1r^{A316T/A316T}* mutants highlights specific molecular alterations that likely contribute to changes in β -cell function. Enhanced expression of *Mafa*, and a less pronounced increase in *Kcnj11* and *Ins1* expression in these mutants, points to potential mechanisms underlying the observed augmented β -cell function but does not explain the change in α -cell function and mass as glucagon expression remains unaffected.

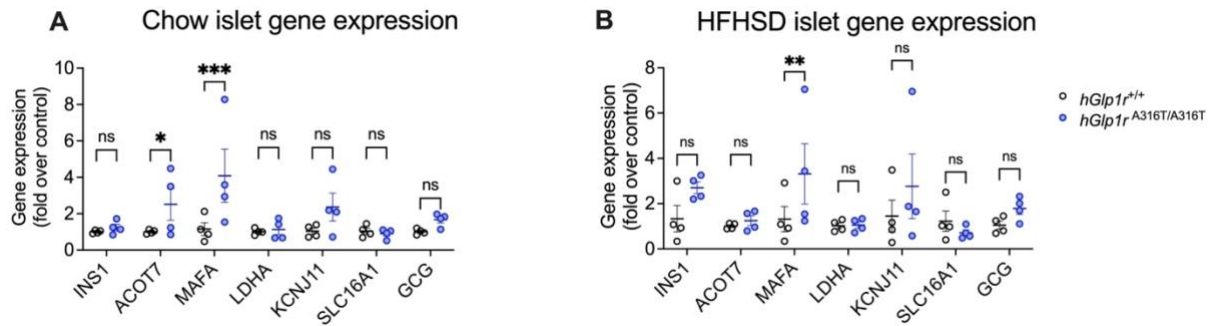


Figure 5.8 Gene expression profiles associated with β -cell identity are altered in $hGlp1r^{A316T/A316T}$ mice under chow and HFHSD conditions. Relative gene expression of selected β -cell “enriched” and “disallowed” genes in $hGlp1r^{A316T/A316T}$ and wild type control mice islets under chow (A) and HFHSD (B) conditions. Comparisons of A316T vs WT controls were made with paired t-tests or two-way analysis of variance (ANOVA) with Sidak's post-hoc tests. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.0001$; ns=not significant. Data are presented as means $\pm$ SEM.

5.2.10 Luxendin645 labelling reveals significantly reduced membrane-localised endogenous GLP-1R expression in islets of $hGlp1r^{A316T/A316T}$ mice

Here, I employed subcutaneous injection of Luxendin645 (exendin-9-Cy5), a fluorescent GLP-1R antagonist probe, followed by transcardial perfusion and harvesting of frozen tissue sections to investigate the endogenous expression of hGLP-1R in pancreatic tissue sections from $hGlp1r^{+/+}$ wild-type and $hGlp1r^{A316T/A316T}$ mutant mice. This approach allowed me to quantify GLP-1R expression *in situ*, providing insights into the receptor distribution and density in the two different genotypes and determining if this matches with previous findings in other *in vitro* models including exogenously expressed receptor in rat pancreatic β -cells (INS-1 832/3) and human pancreatic β -cells (EndoC- β H3). Figure 5.9A shows representative images of pancreatic tissue sections from $hGlp1r^{+/+}$ wild-type and $hGlp1r^{A316T/A316T}$ mutant mice labelled with Luxendin645, as well as DAPI staining for nuclei. The fluorescence intensity, indicative of endogenous GLP-1R expression, was visibly higher in $hGlp1r^{+/+}$ pancreatic tissue sections compared to mutant tissues. Figure 5.9B quantifies the fluorescence

intensity (exendin-9-Cy5/cell) in the pancreatic sections, providing a direct comparison of GLP-1R expression levels between the two genotypes. The results further confirm a significant reduction in GLP-1R expression, particularly at the cell surface in the *hGlp1r*^{A316T/A316T} mutant mice compared to *hGlp1r*^{+/+} wild type-mice (p=0.0264). This significant reduction in GLP-1R surface expression aligns with previous *in vitro* findings in rodent and human cells.

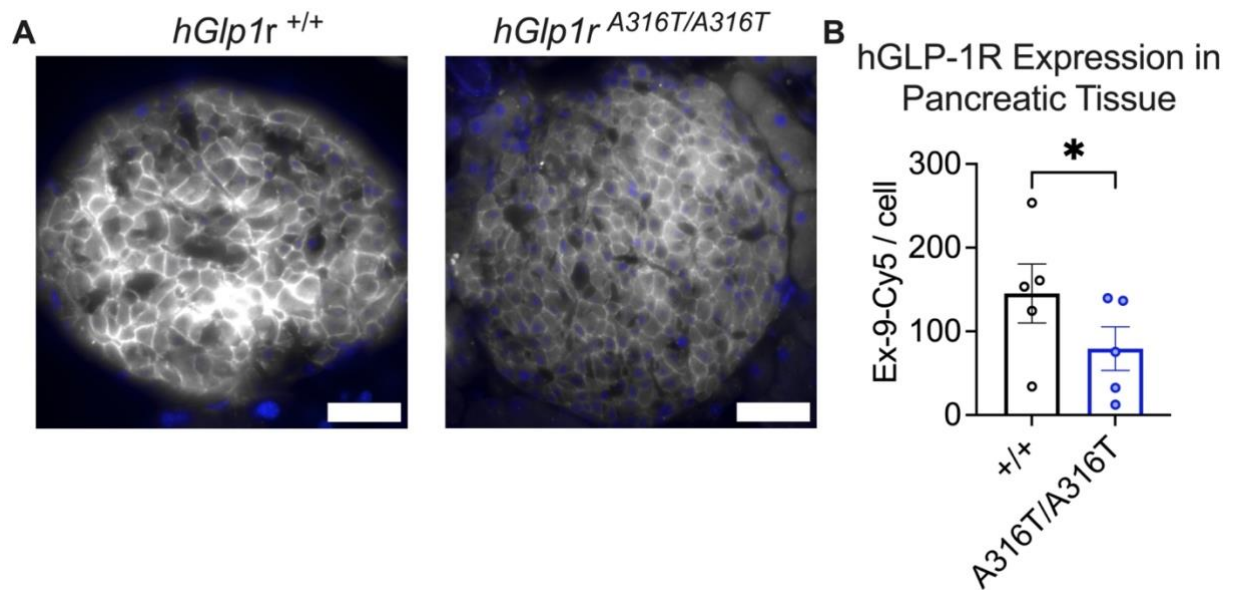


Figure 5.9 The *hGlp1r*^{A316T/A316T} mice exhibit a significant reduction in endogenous receptor cell surface expression. A) Representative fluorescence images of pancreatic islets within pancreatic tissue sections from wild type control *hGlp1r*^{+/+} vs *hGlp1r*^{A316T/A316T} islets subcutaneously administered with the fluorescent GLP-1R antagonist reagent Luxendin-645 (Exendin-9-Cy5) two hours prior to pancreata isolation and processing. Fluorescence for GLP-1R from exendin-9 TMR or Luxendin-645 (white), nuclei (blue) are shown, and bars indicate 50µm. B) Quantification of hGLP-1R surface expression from Luxendin-645 fluorescence signal. alone. Comparisons of A316T vs WT controls islets were made with paired t-tests. *p < 0.05, **p < 0.01, and ***p < 0.0001; ns=not significant. Data are presented as means ± SEM.

5.2.11 The A316T variant expressed in a heterozygous genotype resembles the wild-type phenotype *in vivo*

I have extensively characterised the functional consequences of the presence of the A316T variant in a homozygous state, uncovering significantly improved metabolic parameters including improved glucose tolerance and insulintropic effects, as well as blunted pharmacological responses to GLP-1RAs. However, considering that in the general population, the minor A316T variant allele is more frequently found in a heterozygous state (Table 1.3), often co-existing with other background variants, I aimed to elucidate the phenotypic differences between the heterozygous A316T ($hGlp1r^{A316T/+}$) and the wild-type ($hGlp1r^{+/+}$) genotype, providing insights into how this genetic variation may contribute to metabolic disease susceptibility and altered pharmacological responses. To explore the impact of the heterozygous A316T hGLP-1R variant on glucose homeostasis, I compared various *in vivo* metabolic parameters utilising heterozygous A316T GLP-1R ($hGlp1r^{A316T/+}$) instead of homozygous ($hGlp1r^{A316T/A316T}$) and compared them to wild-type ($hGlp1r^{+/+}$) controls.

Body weight and oral glucose tolerance

There was no significant difference in initial body weights under normal chow conditions and in the rate of weight gain over time on HFHSD between $hGlp1r^{A316T/+}$ and wild-type ($hGlp1r^{+/+}$) controls (Figure 5.10A and 5.11A and B). Similarly, an OGTT showed no significant differences over time or in AUC between the two genotypes both under chow-fed (Figure 5.10B and C) and HFHSD (Figure 5.11B and C). Additionally, fasting glucose levels were also similar between $hGlp1r^{A316T/+}$ and wild-type $hGlp1r^{+/+}$ controls.

Basal and agonist-stimulated glucose levels:

Glucose levels acquired via IPGTTs following vehicle or 2 mol/kg exendin-4 administration at the 0-hour and 6-hour timepoints showed no significant differences between *hGlp1r*^{A316T/+} and wild-type *hGlp1r*^{+/+} controls under chow diet conditions (Figure 5.10D and F). A similar profile is seen with 2 nmol/kg exendin-F1 administration (Figure 5.10I), yet I observe a significant increase in glucose levels at 10 minutes of the 6-hour IPGTT. These findings were confirmed by corresponding AUC analysis (Figure 5.10E, G and J). Moreover, the change in glucose levels Δ AUC from vehicle for each agonist at 0 to 6 hours post-administration was equivalent in the two genotypes (Figure 5.10H and K). Whilst I observed similar findings for vehicle and exendin-F1 stimulation in HFHSD compared to normal chow diet conditions, I observed a significant increase in glucose levels at the 30-minute and 1-hour time-points 6 hours after exendin-4 administration (Figure 5.11E, G, and I); this was corroborated by overall AUC analysis (Figure 5.11F, H and J), showing markedly higher glucose levels at 6-hour compared to acutely in the *hGlp1r*^{A316T/+} versus the wild-type controls (Figure 5.11H). This increase in glucose levels was solely observed for exendin-4, indicative of increased desensitisation with this agonist even under heterozygosity. (Figure 5.11K).

Next, to interrogate whether *hGlp1r*^{A316T/+}-associated glucose levels would still resemble those of mice expressing the wild-type genotype, I performed an analogous study in the same mice cohort of HFHSD-fed animals with the long-acting clinical GLP-1RA semaglutide and the dual GLP-1/GIPRA tirzepatide at 24 and 72 hours after agonist injection. I observed that the *hGlp1r*^{A316T/+} mice exhibited significantly reduced glucose levels compared to the wild-type littermate controls at most time points (10, 30 and 60 minutes) in IPGTTs 24 and 72 hours after vehicle administration, but this marked reduction was less pronounced 72 hours after

vehicle administration, as was confirmed by the AUC comparison (Figure 5.12A and B). On the other hand, differences between the two genotypes were not as pronounced upon 20 nmol/kg semaglutide or tirzepatide administration. In fact, AUC analysis revealed no significant differences between the two genotypes (Figure 5.12 D and F), and glucose curves only small changes in glucose levels with semaglutide and tirzepatide administration (Figure 5.12C and E). Additional Δ AUC analysis to elucidate the agonist-specific effects of the *hGlp1r*^{A316T/+} mice on semaglutide and tirzepatide uncovered that *hGlp1r*^{A316T/+} responses were blunted compared to its own basal responses with both agonists, resembling those of *hGlp1r*^{A316T/A316T} mice (Figure 5.12G and H). Whilst *hGlp1r*^{A316T/+} exhibited worse responses to semaglutide 24 hours after agonist administration (Figure 5.12G), responses to tirzepatide were impaired at both 24- and 72-hours post-agonist administration compared to *hGlp1r*^{+/+} wild-type littermate controls (Figure 5.12H). This can be explained by the differences in wild-type responses to semaglutide versus tirzepatide over 24 and 72 hours. As previously established in the literature, anti-hyperglycaemic responses to tirzepatide are sustained over 72 hours, whereas responses to semaglutide worsen over the same period (469, 471).

Overall, the *hGlp1r*^{A316T/+} mice exhibited a phenotype resembling wild-type littermates in chow and HFHSD conditions, with no enhancement in basal glycaemic control. Yet, prolonged basal responses were improved and responses to the clinical long-acting GLP-1R agonists tirzepatide and semaglutide resembled those of homozygous *hGlp1r*^{A316T/A316T} mice, suggesting that the phenotype of the variant mice extends to heterozygosity when assessed against long-acting GLP-1RAs.

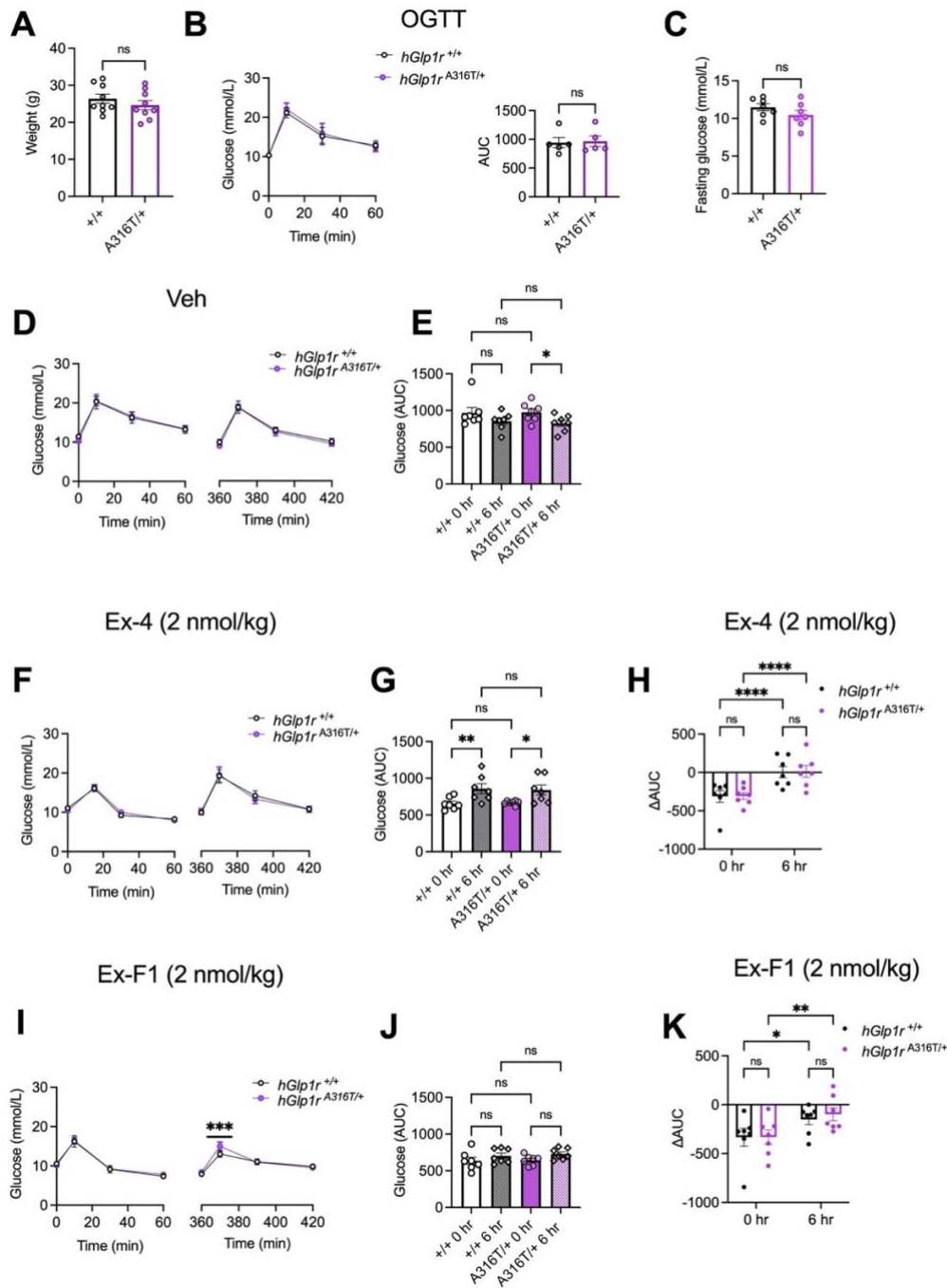


Figure 5.10 Impact of the A316T variant on basal glucose tolerance and metabolic parameters in *hGlp1r*^{+/+} and *hGlp1r*^{A316T/+} heterozygous mice on chow diet. **A)** Initial body weights for *hGlp1r*^{+/+} versus *A316T/+* mixed sex littermate adult mice (n = 9 per genotype) maintained on a chow diet. **B)** OGTT responses and corresponding AUC following 2 g/kg body weight glucose administration via oral gavage after 5 hours of fasting in a mixed sex cohort of adult *hGlp1r*^{+/+} versus *A316T/+* mice on a chow diet (n = 5 per genotype). **C)** Glucose levels after 2 hours

of fasting in a mixed sex cohort of adult *hGlp1r*^{+/+} versus *A316T*^{+/+} mice on a chow diet (n = 7 per genotype). **D, E** IPGTTs (2 g/kg body weight glucose) following acute (0 hours), or 6 hours post-intraperitoneal administration of saline vehicle (Veh) in chow-fed *hGlp1r*^{+/+} versus *A316T*^{+/+} mixed sex adult mice. Glucose curves (D) and corresponding AUCs (E) shown; n = 7 mice per genotype. **(F, G and I, J)** As in (D, E) following administration of 2 nmol/kg exendin-4 (Ex-4) (**F,G**), or exendin-F1 (Ex-F1) (**I,J**). **H,K** Vehicle-corrected Δ AUC responses from (**G**) and (**J**), respectively. Data are mean $\pm$ SEM; *p<0.05; **p<0.01; *** p<0.001; ****p<0.0001; ns, not significant by paired t-tests, one or two-way ANOVA with Sidak's post-hoc tests.

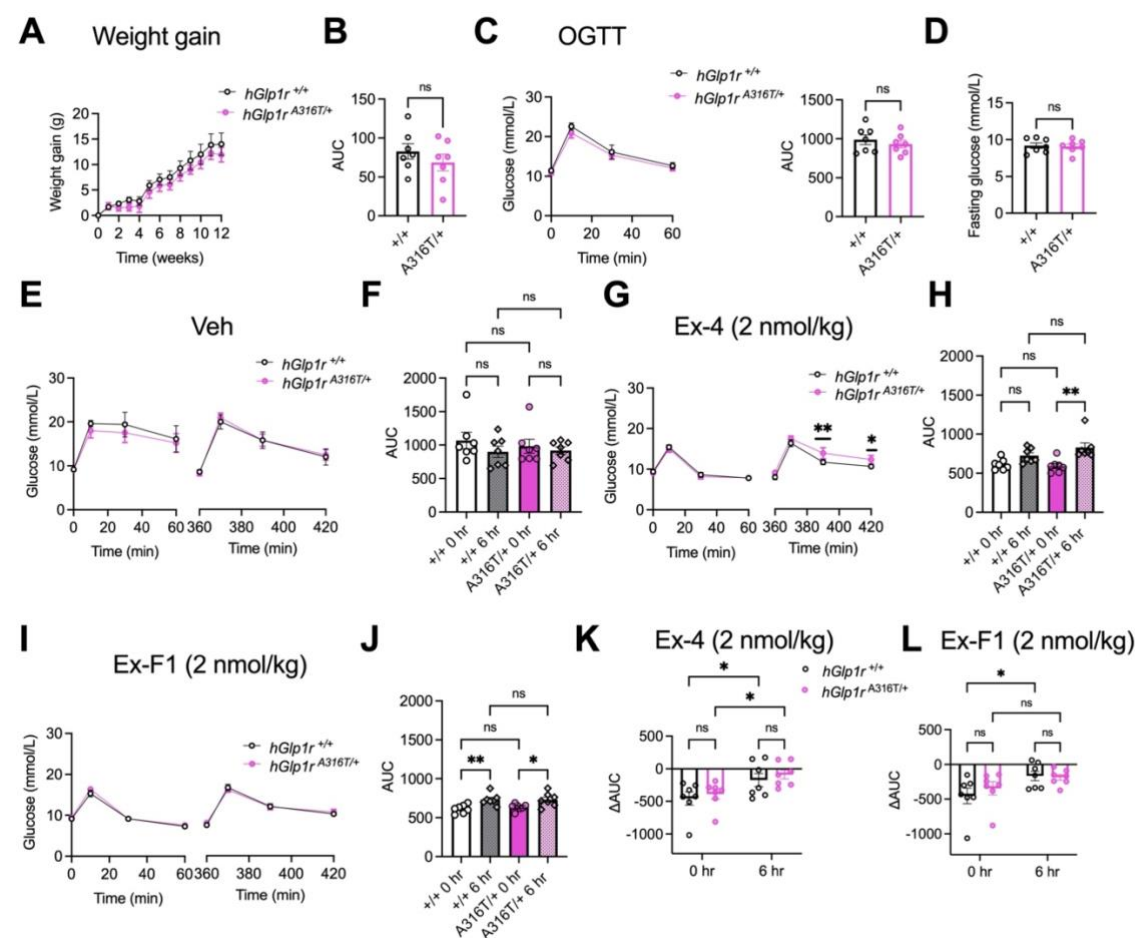


Figure 5.11 Impact of the A316T variant on basal glucose tolerance and metabolic parameters in *hGlp1r*^{+/+} and *hGlp1r*^{A316T/+} heterozygous mice on HFHS diet. **A)** Body weight gain for *hGlp1r*^{+/+} versus *A316T*^{+/+} mixed sex littermate adult mice (n = 7 per genotype) maintained on a HFHS diet for the indicated times and corresponding AUCs (**B**). **(C)** OGTT responses and corresponding AUC following 2 g/kg body weight glucose administration via oral gavage after 5 hours of fasting in a mixed sex cohort of adult *hGlp1r*^{+/+} versus *A316T*^{+/+} mice on a chow diet (n = 5 per genotype). **D)** Glucose levels after 2 hours of fasting from mice

in (A). E, F) IPGTTs (2 g/kg body weight glucose) following acute (0 hours), or 6 hours post-intraperitoneal administration of saline vehicle (Veh) in chow-fed *hGlp1r*^{+/+} versus *A316T*^{+/+} mixed sex adult mice. Glucose curves (E) and corresponding AUCs (F) shown; n = 7 mice per genotype. G,H and I,J) As in (E, F) following administration of 2 nmol/kg exendin-4 (Ex-4) (G,H) or exendin-F1 (Ex-F1) (I,J). H,K) Vehicle-corrected Δ AUC responses from (H) and (J), respectively. Data are mean $\pm$ SEM; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; ns, not significant by paired t-tests, one or two-way ANOVA with Sidak's post-hoc tests.

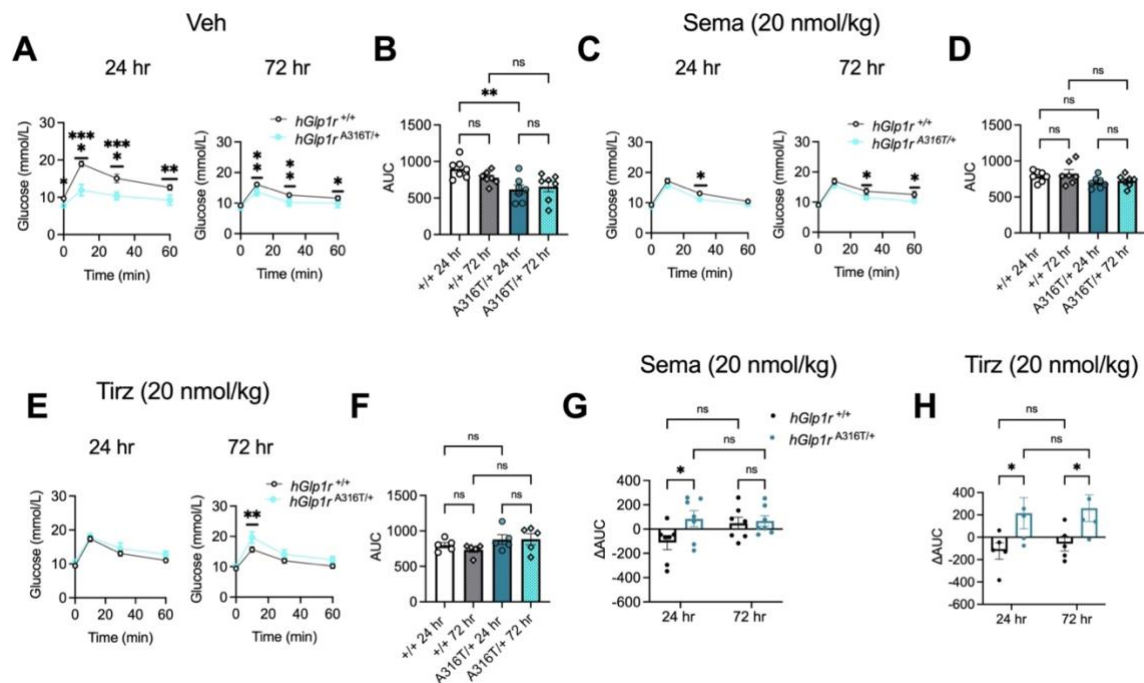


Figure 5.12 *In-vivo* long-acting clinical agonist-induced GLP-1R responses in *hGlp1r*^{+/+} and *hGlp1r*^{A316T/+} heterozygous mice on HFHS diet. Glucose curves and corresponding AUCs following vehicle (Veh) (A, B), 20 nmol/kg semaglutide (Sema) (C, D) or Tirzepatide (Tirz) (F and G) treatment of HFHS-fed *hGlp1r*^{+/+} versus *A316T*^{+/+} mice; IPGTTs performed 24- and 72-hours post-agonist administration; n = 7 mice per genotype. E and H) Vehicle-corrected Δ AUC responses from (D and G). Data are mean $\pm$ SEM; *p<0.05; **p<0.01; ***p<0.0001; ns, not significant by paired t-tests, one- or two-way ANOVA with Sidak's post-hoc tests.

5.3 Discussion

The aim of the work described in this chapter was to design and generate a genetically edited humanized GLP-1R A316T knock-in mouse model and characterise this mouse in a whole-

body context. In doing this I can elucidate the physiological influence of the rs10305492 mutation and ascertain if the GoF effects *in vitro* translate to improved β -cell function and glucose regulation *in vivo*. As I am investigating a human *Glp1r* polymorphism and experiments were conducted in mice, it was important to confirm that the actions seen in the mice would translate into man, and therefore I chose to create the knock-in mouse line utilising the *hGlp1r*^{+/+} parent line obtained from Taconic Biosciences (621) which allowed me to model the human genetic variant. Albeit, while this mouse model was useful for my studies, it possesses an inherent design-based issue related to the receptor level of expression. In the creation of this model, the human *Glp1r* cDNA was inserted downstream of exon 1 of the mouse *Glp1r* gene, which allows the human receptor to be expressed as a mini-gene under the control of the mouse *Glp1r* promoter (Supplementary Figure 2A) (621). Consequently, this modification could potentially result in a reduction in the total expression of the functional human *Glp1r* (Supplementary Figure 2B). Rather than the approach taken by Taconic, a better mouse model would incorporate the full gene including regulatory sequences and introns, to provide more natural regulation of the human *Glp1r* gene and ensure that the gene is expressed at appropriate levels consistent with physiological conditions.

I present here for the first-time a CRISPR/Cas9 transgenic mouse model with a knock-in of the human GLP-1R A316T variant. I assessed metabolic health parameters including body weight, fasting and fed glucose levels, glucose tolerance, pharmacological responses to GLP-1RAs, and plasma insulin levels. Across all *in vivo* experiments, mice were carefully habituated to handling and injections prior to metabolic testing. This likely contributed to the high reproducibility, robustness and low variability of glucose tolerance curves observed in both oral and intraperitoneal GTTs, enhancing the resolution of the genotype-and treatment-

dependent effects. Overall, this characterisation enabled me to determine how the mutation affects insulin secretion and glucose homeostasis in a whole-body context, it also provided important insights into the efficacy of GLP-1R pharmacological therapies in the presence of the mutation. I show that presence of this mutation in a homozygote genotype promotes significant metabolic benefits, including enhanced insulinotropic effects that improve glucose control and clearance.

5.3.1 Investigating metabolic health and glucose profile in *hGlp1r^{A316T/A316T}* and *hGlp1r^{+/+}* wild type mice under normal chow and HFHSD

In the *in vivo* investigation, I monitored body weight and glucose profiles (fasting, fed and 1-hour glucose) in a mixed cohort of *hGlp1r^{A316T/A316T}* and *hGlp1r^{+/+}* wild-type littermate controls under both chow and HFHSD conditions. Results indicated no difference in body weight between the two genotypes, suggesting that differences in weight have no effect on the improved glycaemic control and T2D protection associated with this mutation. Yet, I observed significant reduction in fed, fasting and 1-hour glucose levels in *hGlp1r^{A316T/A316T}* mice compared to wild-type controls, suggesting enhanced glucose regulation intrinsic to the mutation. OGTTs revealed markedly decreased glucose levels at multiple time points, confirming the superior glucose tolerance profile promoted by this mutation. These findings suggest that presence of the A316T variant in a homozygous genotype enhances the incretin effect (endogenous GLP-1 response) and glucose management consistent with GWAS findings in humans (43, 518, 522) as well as *in vitro* findings of improved GLP-1-induced signalling in β -cell lines. Further experiments assessing the protective effects associated with the A316T mutation by subjecting the mice to a HFHSD for 14 weeks, showed a protective

effect of the mutation against diet-induced obesity. The *hGlp1r^{A316T/A316T}* mice presented with a lower rate of weight gain compared to wild-type controls in weeks six to eleven, despite reaching a similar final body weight. Additionally, other parameters such as fed-glucose and 1-hour glucose levels were still significantly lower in *hGlp1r^{A316T/A316T}* mice compared to wild-type controls, though fasted glucose levels were no longer reduced as in the chow condition. These results suggest that the mutation confers resistance to diet-induced metabolic stress, enhances glucose tolerance and maintains better glucose homeostasis even under conditions of metabolic challenge.

Action and signalling of the activated GLP-1R in the CNS, particularly in the hypothalamus, suppresses appetite resulting in reduced food intake and body weight elicited by GLP-1RAs. Consequently, the observed resistance to weight gain in A316T mice under HFHSD suggests that the mutation may enhance anorectic effects associated with GLP-1R activation including appetite regulation and energy balance, aligning with its partially active phenotype that mimics the action of an agonist-induced wild-type receptor.

Future studies should include detailed assessments of food intake and feeding behaviour in the *hGlp1r^{A316T/A316T}* mice under both normal and HFHSD conditions, to clarify whether the mutation directly influences appetite and calorie intake. The CNS effects of the A316T mutation, particularly altered signalling in hypothalamic regions known to regulate appetite, will provide insights into the mechanisms underlying this altered weight regulation phenotype. In addition to this, the improved oral glucose tolerance in the *hGlp1r^{A316T/A316T}* mice on both chow and HFHSD suggests that the partially active receptor variant might affect the secretion of GLP-1 or other hormone peptides including GIP and somatostatin from the gut. Thus, future studies should assess plasma GLP-1 levels and receptor expression in the gut to clarify whether

increased basal A316T GLP-1R activity correlates with elevated circulating GLP-1 or other metabolic hormone peptides. Additionally, extending OGTTs beyond 60 minutes and performing the experiments an hour post glucose bolus administration would be valuable for evaluating receptor desensitisation to the endogenous GLP-1, offering insights into the long-term responsiveness of this variant.

The observation of reduced rates of weight gain with the A316T variant, despite reduced *hGlp1r* levels in the mini-gene expressed humanised mouse model used, raises the possibility that the effects of the A316T variant might be much greater in models exhibiting normal *hGlp1r* expression and in humans.

5.3.2 Investigating basal glucose tolerance in *hGlp1r^{A316T/A316T}* and *hGlp1r^{+/+}* mice under normal chow and HFHSD

IPGTTs used to assess intrinsic glucose lowering effects of the A316T variant under both chow and HFHSD conditions, revealed that the *hGlp1r^{A316T/A316T}* mice consistently exhibited lower blood glucose levels immediately after glucose administration in both conditions. While this phenotype was similar at the 6-hour mark in the chow-fed animals, it was less pronounced compared to HFHSD conditions. These results confirm that the presence of the A316T variant alone resembles that of an agonist-activated GLP-1R wild-type receptor enhancing glucose tolerance and metabolic response even under metabolic stress conditions. The acute and long-term findings suggest effects of the partially active A316T GLP-1R on insulin secretion and glucose clearance.

5.3.3 Investigating pharmacological glucose responses to GLP-1RAs in *hGlp1r^{A316T/A316T}* and *hGlp1r^{+/+}* mice under chow and HFHSD

Interestingly, despite the observed enhanced basal metabolic effects of the A316T variant, agonist-induced responses were impaired in the presence of this variant. Findings from chow IPGTTs with agonist treatments including exendin-4 and exendin-F1, showed that acute and long-term responses to both agonists were blunted compared to the wild-type. Additionally, long-term responses to exendin-4 were impaired in *hGlp1r^{A316T/A316T}* mice, becoming worse than responses to vehicle treatment alone. Conversely, exendin-F1 maintained its glucose-lowering effect in the mutant mice at 6-hours post-treatment, outperforming exendin-4 in the variant mice. This superior long-term glucose lowering effect of exendin-F1, compared to exendin-4, suggests that this agonist induces more favourable conformational changes and sustained receptor activation for the A316T variant, which might be related to this biased agonist profile away from β -arrestin recruitment in wild type animals (357). A similar pharmacological profile was observed under HFHSD conditions, where again responses to both exendin-4 and exendin-F1 were attenuated in *hGlp1r^{A316T/A316T}* mice compared to wild-type animals. Additionally, unlike under chow diet conditions, long-term effects of both agonists were significantly diminished in mutant animals, exhibiting worse responses compared to vehicle treatment alone.

Ultimately, these findings suggest that the A316T mutation enhances inherent glucose regulation but exhibits impaired long-term responses to GLP-1R agonists, exendin-4 under both chow and HFHSD conditions, and exendin-F1 only under metabolic stress. The reduced long-term efficacy of these agonists under metabolic stress conditions in the *hGlp1r^{A316T/A316T}*

mice suggests complex interactions between the genetic mutation, receptor dynamics, and the metabolic environment. These findings highlight the need for further research to better understand these mechanisms to optimise GLP-1R-targeted therapies for individuals with this mutation, especially those with metabolic disorders. Given that the current study administered only a single dose (2 nmol/kg) of GLP-1RAs, future experiments should explore a range of doses to better understand the dose-dependent efficacy, determining the most effective dose that maximises glucose-lowering effects while minimising potential side effects. Use of a high dose could be leading to quicker receptor desensitisation, particularly under metabolic stress, whereas lower doses might maintain prolonged receptor sensitivity.

Intriguingly, HFHS diet feeding did not result in the expected impairment in glucose tolerance in either genotype. This model-specific resistance to diet-induced glucose intolerance may reflect protective effects conferred by the human GLP-1R in the mouse model, which may preserve insulintropic signalling under metabolic stress conditions. This suggests that GLP-1R signalling may be more resilient in this humanised context and could help explain the preserved glycaemic control observed in vivo, even under chronic nutritional challenge.

5.3.4 Investigating pharmacological glucose responses to long-acting GLP-1RAs in *hGlp1r^{A316T/A316T}* and *hGlp1r^{+/+}* wild-type mice under HFHSD

Analogous experiments investigating the effects of the mutation on responses to clinically leading GLP-1RAs semaglutide and tirzepatide also revealed a similar phenotypic profile to those observed for the other short-acting GLP-1RAs. Under vehicle treatment, the *hGlp1r^{A316T/A316T}* mice exhibited improved glucose tolerance at both 24 and 72 hours compared to wild-type controls. On the other hand, consistent with the agonist-induced profile elicited by the A316T mutation, despite semaglutide administration lowering glucose levels in the

mutant mice at 24 hours, overall glucose regulation was impaired compared to wild-type mice. Conversely, tirzepatide did not alter glucose levels in the *hGlp1r*^{A316T/A316T} mice, and worsened glucose levels compared to vehicle treatment, highlighting a marked impairment in glucose regulation with this balanced GLP-1R:GIPR agonist. This may reflect receptor desensitisation or altered intracellular signalling dynamics in a system already biased toward enhanced GLP-1R activity. Given that the A316T mutation enhances receptor function, additional stimulation by a dual agonist may overwhelm regulatory mechanisms, leading to suboptimal insulin secretion timing or reduced receptor responsiveness. Alternatively, tirzepatide's GIPR agonism or non-canonical signalling may interfere with optimal GLP-1R-driven glucose regulation in this sensitised genetic background.

Overall, my results bolster the characterisation of the A316T as a GoF mutation that enhances intrinsic glucose regulation but impairs incretin-mediated glucose effects, which might be explained by altered conformational arrangements within the A316T GLP-1R structure, specifically in the water network. As before, future studies should trial administration of different doses in the hope of determining an optimal dose that might rescue this phenotype or even trialling the administration of allosteric GLP-1R modulators like BETP and compound 2 (601). In addition to this, future studies should also examine the effect of chronic administration of these clinical agonists on mice possessing the A316T mutation, to determine its effects on other factors such as weight loss in the overall phenotype. It is plausible that either the response is rescued over a longer period of time, or the negative effects are exacerbated resulting in increased metabolic stress and even diabetic progression. On that point, in this study I did not investigate the effect of long-lasting agonists under chow-fed conditions, which might elicit a different A316T associated phenotype. It would be interesting

to find out if the impaired phenotype I observe under diet-induced obesity persists in lean animals.

Another limitation is the reduced *in vivo* potency of semaglutide and tirzepatide observed in our humanised GLP-1R mouse model, despite their known high clinical efficacy, in both genotypes. This may reflect limitations imposed by reduced cell surface human GLP-1R expression, as shown by Exendin-9-TMR labelling, rather than intrinsic deficiencies in the ligands themselves. Since both long-acting agonists require sufficient receptor density for optimal signalling, reduced surface receptor levels may limit their pharmacological action. This highlights that receptor expression and not just ligand affinity plays a critical role in determining the magnitude of *in vivo* drug effects, and that futurized humanised models should consider integrating full genomic loci to preserve regulatory and expression dynamics.

5.3.5 *In vivo* assessment of insulin secretion in *hGlp1r*^{A316T/A316T} and *hGlp1r*^{+/+} mice

To determine if the enhanced glucose tolerance observed in *hGlp1r*^{A316T/A316T} mice was due to increased insulinotropic effects, as observed in human pancreatic β -cells, plasma insulin levels at the 10-minute mark post-glucose and agonist administration during both acute (0hr) and long-term (6 hr) IPGTTs were assessed. This time-point was selected as it corresponds to that where the most significant differences between genotypes were noted. As was foreseeable, vehicle administration alone increased plasma insulin levels in the *hGlp1r*^{A316T/A316T} mice compared to wild-type controls acutely and this was sustained over a longer period. On the other hand, consistent with the glucose tolerance findings, exendin-4-induced insulinotropic effects were reduced in mutant mice relative to wild-types, particularly over prolonged periods of time, where they diminished compared to vehicle responses alone. The inherent ability of

the A316T mutation to elevate insulin levels is maintained under HFHSD conditions, contributing to enhanced glucose tolerance even under metabolic stress.

Ultimately, the A316T mutation enhances glucose tolerance primarily through improved insulinotropic effects but impairs responses to GLP-1RAs. Despite having collected the blood samples, due to time and cost constraints I did not investigate the plasma insulin levels in response to exendin-F1, and agonist-induced responses in general in HFHSD animals. Future studies should do this to ascertain if indeed the agonist associated phenotype observed in IPGTTs is also explained by altered insulinotropic effects.

5.3.6 Investigating alterations in islet cell identity and gene expression in the presence of the A316T variant

The composition and spatial arrangement of pancreatic islet cells, primarily α - and β -cells, are critical for maintaining glucose homeostasis. Alterations in the relative mass or spatial arrangement of these cells can alter glucose regulation, contributing to protection from or progression of metabolic disorders. Previous studies also highlight the effect of GLP-1R activation via agonists on β -cell mass, but their impact on α -cell mass remains unclear. In this study I aimed to investigate the effects of the A316T variant on islet cell composition as another underlying mechanism to explain enhanced insulinotropic and glucose regulation effects, in both normal and metabolic stress conditions. Given the partially active phenotype of the A316T variant, I predicted that this variant would also increase β -cell mass, resembling an activated GLP-1R. Contrary to what I hypothesised, no difference was observed in islet diameter, β -cell mass, α -cell mass, or α -to- β -cell ratio between the genotypes under chow

diet. This suggests that the mutation does not markedly alter islet cell composition under normal dietary conditions or during embryonic development. Yet, remarkably, under HFHSD conditions, while β -cell mass remained unchanged, I observed a marked increase in α -cell mass and therefore α -to- β cell ratio in *hGlp1r^{A316T/A316T}* mice tissue compared to wild-type controls. Additionally, I observed a notable change in the intra-islet localisation of α -cells, with a significant proportion localised within the core of islets rather than restricted to the periphery, resembling human islet architecture. This mislocalisation suggests a disruption of normal islet cytoarchitecture, which may result from a combination of intrinsic and extrinsic factors such as endothelial cells and islet microenvironment influenced by the A316T GLP-1R. Islet architecture, including peripheral positioning of α -cells, is established during embryonic development but remains plastic in adult life, particularly under metabolic stress or injury (622-626). In the context of the *hGlp1r^{A316T/A316T}* mice, it is plausible that enhanced basal GLP-1R signalling alters the expression or secretion of morphogenetic cues or guidance molecules (e.g., ECM components, integrins, semaphorins) that normally guide α -cell positioning during islet remodelling (627-629). Alternatively, altered paracrine signalling due to heightened β -cell activity could impact α -cell adhesion, migration, or repulsion, particularly if the local balance of insulin, somatostatin, or GLP-1 is disrupted (627, 629).

Another plausible contributor is the presence of bi-hormonal cells, which is evident in A316T islets and may reflect a transitional or de-differentiated state (622-624, 630). These cells may adopt atypical spatial patterns, thereby disrupting the normal α -/ β cell segregation. Future experiments such as immunostaining for adhesion and polarity markers (e.g. E-cadherin, β -catenin, ZO-1) or lineage tracing of α -to - β transitions could help resolve the cellular basis of this mislocalisation.

Interestingly, the V369M naturally occurring mutation in GCGR, also led to improved glucose tolerance and pronounced α -cell hyperplasia (510, 511). This phenotype supports the concept that defective glucagon signalling can drive adaptive α -cell hyperplasia, as a compensatory response to disrupted glucagon receptor activity. This α -cell expansion may serve to increase glucagon output to restore hepatic amino acid metabolism and glucose regulation, particularly when normal glucagon signalling is impaired. In the case of the *hGlp1r*^{A316T/A316T} mice, it is plausible that enhanced GLP-1R activity alters intra-islet paracrine networks or systemic glucagon signalling feedback loops, inadvertently mimicking a state of glucagon resistance or suppression.

Moreover, α -cells are increasingly recognised as metabolically responsive and plastic. Beyond their canonical role, they are capable of transdifferentiating into insulin-producing β -cells or serving as a source of proglucagon-derived peptides such as GLP-1 particularly under metabolic stress or injury (624). Chambers *et al.* showed that pancreatic α -cell derived GLP-1, not intestinal GLP-1, significantly contributes to GLP-1R function and glucose metabolism, as well as mediating beneficial responses to DPP-IV inhibitors (230). On the other hand, recent studies have shown that insulin secretion is co-modulated by both GLP-1R and GCGR signalling under basal glucose conditions, whereas under high glucose conditions, glucagon primarily acts through GLP-1R (620). This shows another layer of complexity to the α -cell paracrine signalling hypothesis, as while GLP-1 is a 300-fold more potent GLP-1R agonist, glucagon is present at much higher levels in healthy islets (631). This may become functionally relevant in the context of receptor variants such as A316T that alter GLP-1R sensitivity to glucagon.

These findings raise important questions about how GLP-1R variants may alter α -cell behaviour, identity, and adaptive responses under metabolic stress conditions. The α -cell hyperplasia observed in HFHSD *hGlp1r*^{A316T/A316T} mice could reflect a compensatory response to altered glucagon signalling, similar to that seen in GCGR-deficient models. Future studies should investigate α -cell derived GLP-1 secretion and plasma GLP-1 levels collected during OGTTs, alongside islet immunostaining to examine α -cell proliferation. Together, this would determine whether α -cell expansion in the *hGlp1r*^{A316T/A316T} mice islets supports endocrine compensation, β -cell regeneration, or a broader shift in islet cell plasticity.

Gene expression analysis of “enriched” and “disallowed” genes was performed in islets isolated from A316T mice to further understand underlying mechanisms behind the observed differences in islet cyto-architecture and cell composition between the *hGlp1r*^{A316T/A316T} and the *hGlp1r*^{+/+} genotypes under both normal and HFHSD. Under both conditions, I observed no significant differences in the expression of “disallowed” genes *Ldhl* and *Slc16a1* in mutant compared to wild-type islets. Yet, I observed a significant increase in *Acot7* expression in mutant islets only under chow fed conditions. For “enriched” genes, there was a trend for upregulated *Ins1* expression, but notably *Mafa* expression was significantly elevated in *hGlp1r*^{A316T/A316T} islets under both chow and HFHSD. This suggests that while the mutation does not affect insulin gene expression directly, it may indirectly influence it via upregulation of *Mafa*. Additionally, I also observed a trend towards increased expression of *Kcnj11* expression under both dietary conditions, potentially contributing to improved glucose tolerance and insulinitropic effects of this variant.

Together the increased expression of *Mafa* and the trend towards higher *Kcnj11* and *Ins1* expression support the notion of augmented β -cell insulin secretion in the presence of the mutation. However, *Gcg* expression was not increased significantly, suggesting that the changes in α -cell mass observed are not translated to increased expression of glucagon or could not be detected by lack of power in the qPCR experiments. These findings provide important insights into how altered GLP-1R activity impacts islet cell identity, composition, and function, as well as identifies molecular factors which are regulated by GLP-1R activation such as *Mafa*, *Kcnj11* and *Acot7*. By hydrolysing acyl-CoA, ACOT7 (Acyl-CoA Thioesterase 7) facilitates the mobilisation of free fatty acids, which are necessary for phospholipid biosynthesis, and are also used in β -oxidation to produce energy (632). Remarkably, the regulatory role of ACOT7 in maintaining lipid homeostasis aligns with the interactome profile association with the A316T mutation, further corroborating the importance of these processes in GLP-1R function. This also suggests that the mutation could lead to an increased metabolic rate or altered energy demands within the islets, necessitating efficient fatty acid metabolism. Further studies are warranted to investigate this interesting effect.

Nonetheless, one limitation of this approach is that qPCR to assess altered gene expression were performed on RNA isolated from whole unsorted islets, which contain various cell types including α , β , δ and PP cells. Therefore, without FACS, RNA extracted from whole islets includes contributions from all these cell types, potentially confounding the expression signals specific to each cell type. “Enriched genes” which are highly expressed in β -cells might not show significant differential expression as β -cell populations are not adequately isolated, masking true biological changes. Similarly, the expression of “disallowed genes”, which are

typically inhibited in β -cells but expressed in other cell types, may appear higher due to contributions from non- β -cells, leading to incorrect conclusions about their regulation.

5.3.7 *In vivo* assessment of endogenous GLP-1R expression *hGlp1r*^{A316T/A316T} and *hGlp1r*^{+/+} mice

Finally, I confirmed that the substantial reduction in cell surface receptor expression associated with the A316T mutation is also present when I investigate the endogenous expression of the receptor using tissue from *hGlp1r*^{A316T/A316T} mice in conjunction with Luxendin645, a fluorescent GLP-1R antagonist probe. This finding is consistent with the *in vitro* studies conducted on INS-1 832/3 and EndoC- β H3 cell models. This further corroborates the enhanced basal lysosomal trafficking, turnover and signalling of the A316T receptor, and so its inherent GoF phenotype, which is consistent across several models. This approach allowed me to visualise and quantify endogenous GLP-1R expression in pancreatic tissue, but it can also be used in the same way to investigate altered endogenous GLP-1R expression in other tissues such as the brain, gut and heart.

5.3.8 *In vivo* assessment of metabolic and glycaemic control in *hGlp1r*^{A316T/-} and *hGlp1r*^{+/+} mice

Given that the minor A316T variant allele is more frequently found in a heterozygous state (Table 1.3), and often coexists with other *Glp1r* variants, I aimed to elucidate the phenotypic differences between the heterozygous A316T (*hGlp1r*^{A316T/+}) and the wild-type (*hGlp1r*^{+/+}) genotype, providing insights into how this genetic variation may contribute to metabolic disease

susceptibility and altered pharmacological responses in humans. The findings of this study revealed that heterozygous *hGlp1r*^{A316T/+} mice exhibit a phenotype largely similar to the wild-type littermates under both normal chow and HFHSD diet conditions, with basal metabolic parameters such as body weight, weight gain, and fasting glucose levels remaining unaffected by the presence of one minor allele. However, pharmacological efficacy of GLP-1RAs was altered. The blunted responses to semaglutide in *hGlp1r*^{A316T/+} mice, resembling the homozygous *hGlp1r*^{A316T/A316T} phenotype, suggest that even heterozygosity for this variant can significantly impact drug responses. This is particularly relevant given the sustained anti-hyperglycaemic responses to tirzepatide observed in wild-type mice, which were not mirrored in the heterozygous variant carriers. It is also important to note that while I observed a significant reduction in glucose levels after 24 hours of vehicle administration in HFHSD-fed *hGlp1r*^{A316T/+} mice compared to wild-type counterparts, this might be due to the longer duration these animals were on a HFHS diet, suggesting that the effects of the A316T variant become apparent under metabolic stress potentially due to the reduced function of the WT receptor in these conditions. It is also encouraging that these findings in *hGlp1r*^{A316T/+} mice are mirrored by human studies mentioned in section 1.6.4, where they did not observe any differences in incretin effect or glucose control between heterozygous *hGlp1r*^{A316T/+} and wild-type individuals. However, these studies did not include any experiments assessing pharmacological responses to GLP-1RAs, which would have been interesting for comparison if available, to confirm phenotypes observed in my animal models.

Another thing to consider is the fact that individuals that are heterozygous for this variant, possess one wild-type and one variant allele. This genetic configuration does not necessarily result in the production of each receptor type in equal proportions. The actual expression levels

can be influenced by several factors, including allelic expression imbalance, regulatory elements, mRNA stability, and protein translation efficiency. This variability in receptor expression levels may explain phenotypic differences between the homozygous and heterozygous mice. In homozygous mice, only the A316T receptors are present, leading to a more pronounced and consistent phenotypic expression of the variant, while heterozygous mice will possess a mix of WT and A316T receptors, resulting in a phenotype that can range from normal to partially affected, depending on the relative expression levels of each receptor type. For instance, the blunted pharmacological responses to GLP-1RAs observed in *hGlp1r*^{A316T/+} mice, may be due to a dominant-negative effect where the presence of A316T receptors interferes with the function of WT receptors. This interference could vary depending on the proportion of these receptors, leading to differences in drug efficacy and metabolic responses between heterozygous and homozygous individuals.

Given the phenotypic differences observed between *hGlp1r*^{A316T/A316T} and *hGlp1r*^{A316T/+} genotypes, future studies should investigate the potential for hetero-oligomerization between WT and A316T receptors. Such studies could involve co-immunoprecipitation experiments to detect physical interactions between WT and A316T receptor proteins in heterozygous cells. Additionally, fluorescence resonance energy transfer (FRET) or bioluminescence resonance energy transfer (BRET) assays could be employed to quantify the proximity and interaction dynamics between the different receptor variants in live cells. Understanding whether and how these receptors form hetero-oligomers could help elucidate the mechanisms by which the A316T variant influences overall receptor function and pharmacological responses. These insights could further inform targeted therapeutic strategies and the development of drugs that

specifically modulate the activity of receptor oligomers, enhancing treatment efficacy for individuals with the A316T variant.

Chapter 6: Influence of the rs10305492 variant in murine and human islet models

6.1 Introduction

The *in vitro* studies indicate that the A316T variant alters the membrane localisation, trafficking, signalling profile as well functional outcomes of GLP-1R in both rodent and human pancreatic β -cells. The changes observed in GSIS, trafficking and $G\alpha_s$ recruitment strongly suggest that the variant exhibits a GoF phenotype promoting enhanced glucose homeostasis independent of agonist stimulation, and that in fact exogenous agonist administration specifically can attenuate the intrinsic beneficial effects that the variant has on pancreatic β -cell function. Following these studies, further investigations into the role of the A316T variant in glucose homeostasis and its dynamic responses to various stimuli such as glucose, GLP-1RAs and diet in a whole-body context using a genetically engineered knock-in mouse model were conducted. In parallel to the *in vitro* studies, *in vivo* studies confirmed that the variant promotes enhanced glucose homeostasis, glucose tolerance, and insulin secretion independent of agonist treatment both under chow and HFHSD conditions. This matches the phenotypes associated with the variant in GWAS studies as well as characteristics of enhanced activation and enhanced β -cell function observed *in silico* and *in vitro*.

After this, I sought to bridge the gap between physiological relevance observed *in vivo* and the mechanistic cellular insights observed *in vitro*, by validating my findings on the A316T variant in *ex vivo* murine islets from the knock-in mice, as well as by using islets from a *hGlp1r* knock-

out model that I generated, as well as in human donor islets, which were transduced with the previously described SNAP-hGLP-1R^{WT} and -A316T adenoviral vectors. Investigating the influence of the A316T variant in islets allowed me to more precisely control experimental conditions as islets are removed from the whole organism enabling me to investigate specific cellular and molecular pathways involved without the confounding factors of systemic physiology such as neural input, hormonal signalling, and interactions with other organs that may be altering glucose metabolism and pharmacological responses. By utilising this approach, I can provide a more precise understanding of how the A316T variant alters islet function as well as to connect the structural, molecular and cellular mechanisms with systemic physiological outcomes observed *in vivo*.

The *hGlp1r* knock-out mouse line was generated as a byproduct of my CRISPR/Cas9 strategy to knock-in the A316T mutation into the *hGlp1r*^{+/+} mice, whereby due to the reduced efficiency of HDR-mediated repair in comparison to NHEJ repair, the Cas9 generated DSB is imprecisely repaired by NHEJ creating indels and subsequently frameshift mutations, so that a knock-out mouse line was generated. Pancreatic islets isolated from these mice were transduced with previously described adenoviral vectors, highly efficient at transducing the EndoC-βH3 GLP-1R knock-out cell line, ensuring robust transient expression of the SNAP-tagged A316T variant. Using this model, I could elucidate the role of this variant in pancreatic β-cell function, islet cell signalling, insulin secretion, and pharmacological responses to GLP-1RAs in a primary context.

In this chapter, I will explore the impact of the GLP-1R A316T variant on receptor cell surface expression, signalling pathways, insulin secretion, and glucose regulation in both murine and human islets using *ex vivo* approaches. Through these studies, I aim to uncover the mechanistic

underpinnings that link this specific genetic variant to alterations in regulation of glucose metabolism, including insulin secretion and islet cell function. Such insights are not only foundational for understanding the physiological influences of GLP-1R variants such as the A316T but also for the development of more tailored and effective therapeutic strategies for diabetes management.

6.2 Results

6.2.1 Validation of the *hGlp1r*^{-/-} mice generated as a byproduct of genome editing with CRISPR/Cas9

The *hGlp1r*^{-/-} mouse line was an unintended result of my CRISPR/Cas9 strategy to introduce the A316T mutation into the *hGlp1r*^{+/+} mouse line. The higher efficiency of non-homologous end joining (NHEJ) over homology-directed repair (HDR), whereby cells more frequently repair Cas9-induced DSBs through NHEJ because its quicker and more active during most phases of the cell cycle compared to HDR (only active in the S and G phase), combined with its propensity to generate indels resulted in unintended frameshift mutations and the generation of the *hGlp1r* knockout mouse line. In fact, more than 40% of my litter post-genome editing contained such indels and resulting frameshift mutations as observed from the Sanger sequencing results represented in Figure 6.1A. To further validate this mouse line is indeed a functional knock-out, I further characterised it by labelling the islets with the fluorescent antagonist exendin-9-TMR followed by confocal microscopy, prior to use in subsequent *ex vivo* experiments. As shown in Figure 6.1B, the *hGlp1r* knock-out mouse islets exhibited no

detectable labelling confirming the successful knock-out of GLP-1R in these mice. In contrast, *hGlp1r*^{+/+} islets showed clear labelling, validating the presence of the receptor in these islets. Notably, the observed cytoplasmic fluorescence in *hGlp1r*^{+/+} islets is likely due to basal receptor internalisation, and this is consistent with the GLP-1R being a rapidly internalising receptor, so unless imaging is performed immediately post Ex9-TMR labelling, a significant proportion of surface-localised receptors may already have been endocytosed as seen in Figure 6.1B.

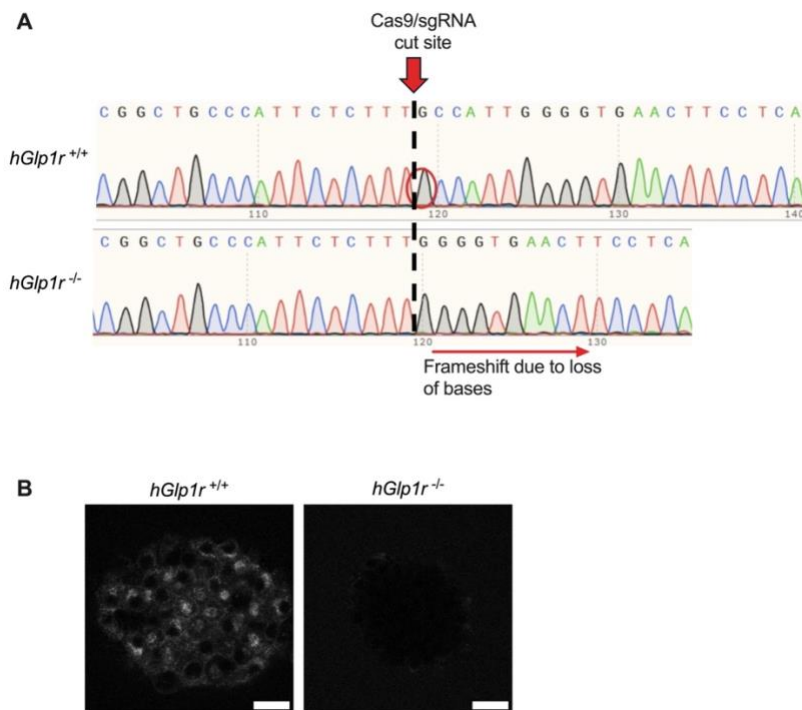


Figure 6.1 Generation of *hGlp1r*^{-/-} mice as a byproduct of CRISPR/Cas9 gene editing. **A)** Representative Sanger Sequencing genotyping results from a *hGlp1r*^{-/-} and a wild-type *hGlp1r*^{+/+} control mice. The red arrow depicts the Cas9/sgRNA cut site and the bases lost because of the indel mutation. **B)** Representative fluorescence image of islets labelled with exendin-9-TMR, shown in white, to detect endogenous hGLP-1R expression in islets from *hGlp1r*^{-/-} and a wild-type *hGlp1r*^{+/+} control mice. Scale bars = 50 μm.

6.2.2 The A316T variant reduces membrane localisation of the human GLP-1R in murine and human islets

As previous *in vitro* experiments showed reduced surface expression and localisation of the A316T GLP-1R compared to the wild-type in both rat and human β -cell lines, I performed further experiments to examine the impact of the A316T mutation on GLP-1R membrane localisation in mouse islets isolated from knock-in $hGlp1r^{+/+}$ or $hGlp1r^{A316/A316T}$, or $hGlp1r^{-/-}$ mice transduced with SNAP-hGLP-1R^{WT} or ^{-A316T} adenoviruses, as well as in human islets transduced with these same adenoviruses. Using exendin-9-TMR, I assessed GLP-1R surface expression in islets isolated from $hGlp1r^{+/+}$ or $hGlp1r^{A316/A316T}$ fed either a standard chow diet or a HFHSD. In chow-fed islets (Figure 6.2A, left), $hGlp1r^{+/+}$ mice exhibited significantly higher GLP-1R surface expression compared to $hGlp1r^{A316/A316T}$ quantified by mean fluorescence intensity in Figure 6.1B ($p=0.0448$). This difference was further exacerbated under HFHSD conditions, where GLP-1R surface expression in $hGlp1r^{+/+}$ remained significantly higher than in the $hGlp1r^{A316/A316T}$ mutants (Figure 6.2A, right; quantified in C, $p=0.0029$).

Next, I explored the cell surface localisation of the GLP-1R using a SNAP-tag labelling technique in $hGlp1r^{-/-}$ mouse and human islets transduced with SNAP-hGLP-1R^{WT} or ^{-A316T} adenoviruses for 24 hours. I found that mice islets exogenously expressing the A316T receptor, exhibited markedly reduced cell surface expression compared to WT GLP-1R-expressing islets (Figure 6.2F and E, $p=0.0001$). This was consistent in human islets, where the A316T variant also showed significantly lower surface localisation than the WT GLP-1R (Figure 6.2D, lower panels, quantified in 6.2F, $p=0.0473$). These findings demonstrate that the

A316T mutation leads to a significant decrease in cell surface localisation, consistent with previous findings, even when the receptor is expressed endogenously under both dietary conditions, and exogenously in both murine and human islets.

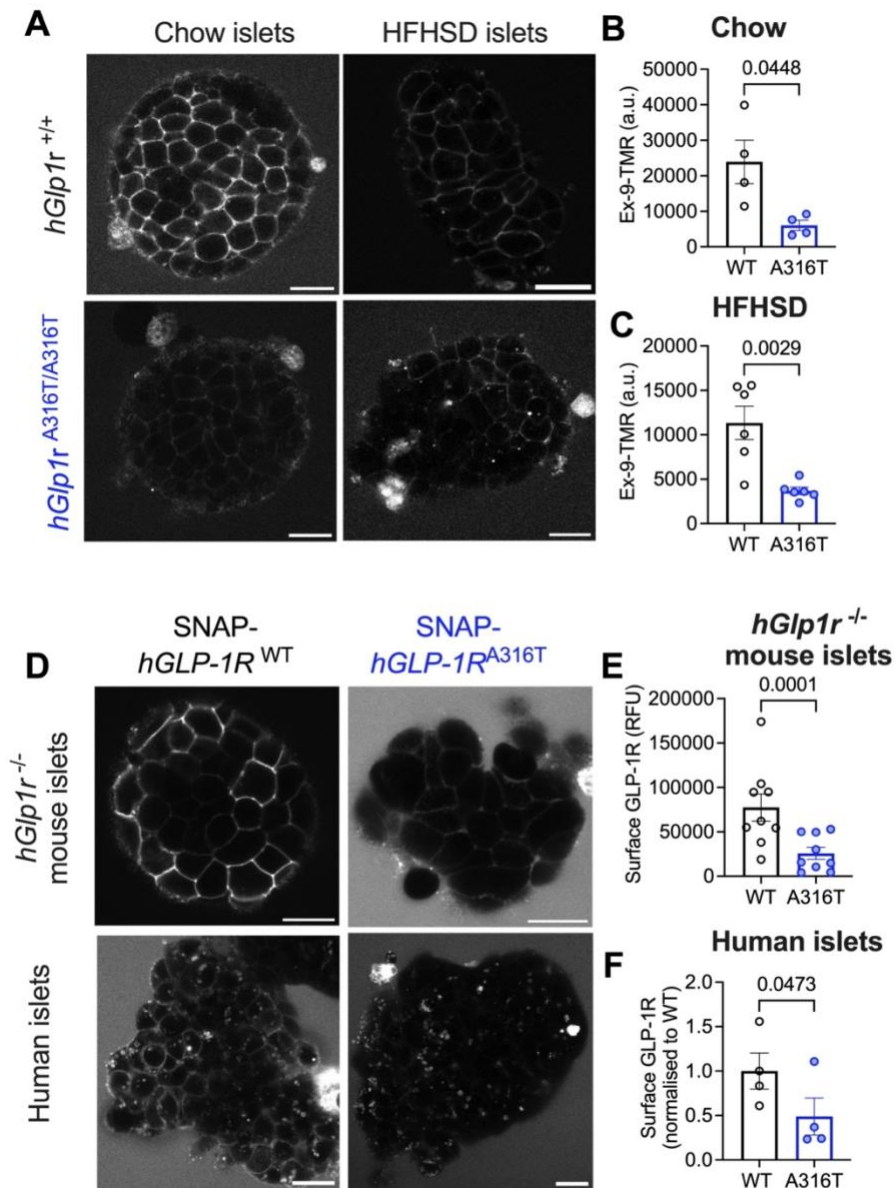


Figure 6.2 The A316T variant leads to reduced endogenous and heterogenous receptor cell surface expression in mouse and human islets. **A)** Representative images and fluorescence in islets from *hGlp1r*^{A316T/A316T} vs *hGlp1r*^{+/+} wild type control islets treated with 1μM exendin-9-TMR for 10 minutes in chow and HFHS-diet mice (n=4 mice per genotype). **B and C)** Quantification of endogenous hGLP-1R surface expression from exendin-9-TMR in (A) under chow (**B**) and HFHS (**C**) conditions. **D)** Representative images and

fluorescence in *hGlp1r*^{-/-} mouse (top) and human (bottom) islets transduced with pAV-SNAP-hGLP-1R^{WT} or -A316T labelled with SNAP-Surface Alexa Fluor 647 for 10 minutes. **E and F)** Quantification of SNAP-GLP-1R surface expression from *hGlp1r*^{-/-} mouse and human islets. Fluorescence from exendin-9-TMR or SNAP-Surface Alexa Fluor 647 is shown (white), and bars indicate 50 μ m. Comparisons of A316T *versus* WT were made with paired t-tests. **p* < 0.05, ***p* < 0.01, and ****p* < 0.0001; ns=not significant. Data are presented as means $\pm$ SEM.

6.2.3 The A316T variant exhibits increased basal lysosomal targeting in murine and human islets

To further investigate the effect of the A316T mutation on cell membrane localisation, endocytosis and basal trafficking of the GLP-1R in mouse islets, I extended the bafilomycin lysosomal inhibition experiments that were conducted *in vitro*. First, I compared GLP-1R surface expression in islets isolated from knock-in mice endogenously expressing the WT or A316T GLP-1R variant, in the presence and absence of lysosomal inhibition. I found that, consistent with findings from the previous section, surface GLP-1R expression was significantly reduced in mouse islets from the *hGlp1r*^{A316/A316T} compared to those isolated from WT control littermates (*p*=0.0071), yet treatment with the lysosomal inhibitor bafilomycin restored surface expression in A316T mutant islets, eliminating the difference in cell surface localisation between the two genotypes (Figure 6.3A and B).

Additionally, using *hGlp1r*^{-/-} mouse islets exogenously expressing either SNAP-hGLP-1R^{WT} or A316T, I observed similar trends. Surface GLP-1R expression in SNAP-hGLP-1R^{WT} was significantly higher compared to mice islets expressing SNAP-hGLP-1R^{A316T}, yet this difference was not statistically significant (*p*=0.1874) (Figure 6.3C and D). As before, there was a modest, non-significant improvement in A316T receptor surface expression in response to bafilomycin treatment (*p*=0.6135), reducing the difference in surface expression compared

to the WT receptor (Figure 6.3D). These results corroborate the results obtained using the rat INS-1 832/3 cell line model. Therefore, this data demonstrates that the A316T mutation in GLP-1R consistently reduces cell surface localisation of the receptor across different experimental models, including mouse islets, human islets, and rat pancreatic β -cells. This reduction in surface expression, partially alleviated by lysosomal inhibition with bafilomycin treatment, suggests a potential increased basal trafficking and turnover rate by which the A316T mutation alters GLP-1R function contributing to altered metabolic regulation.

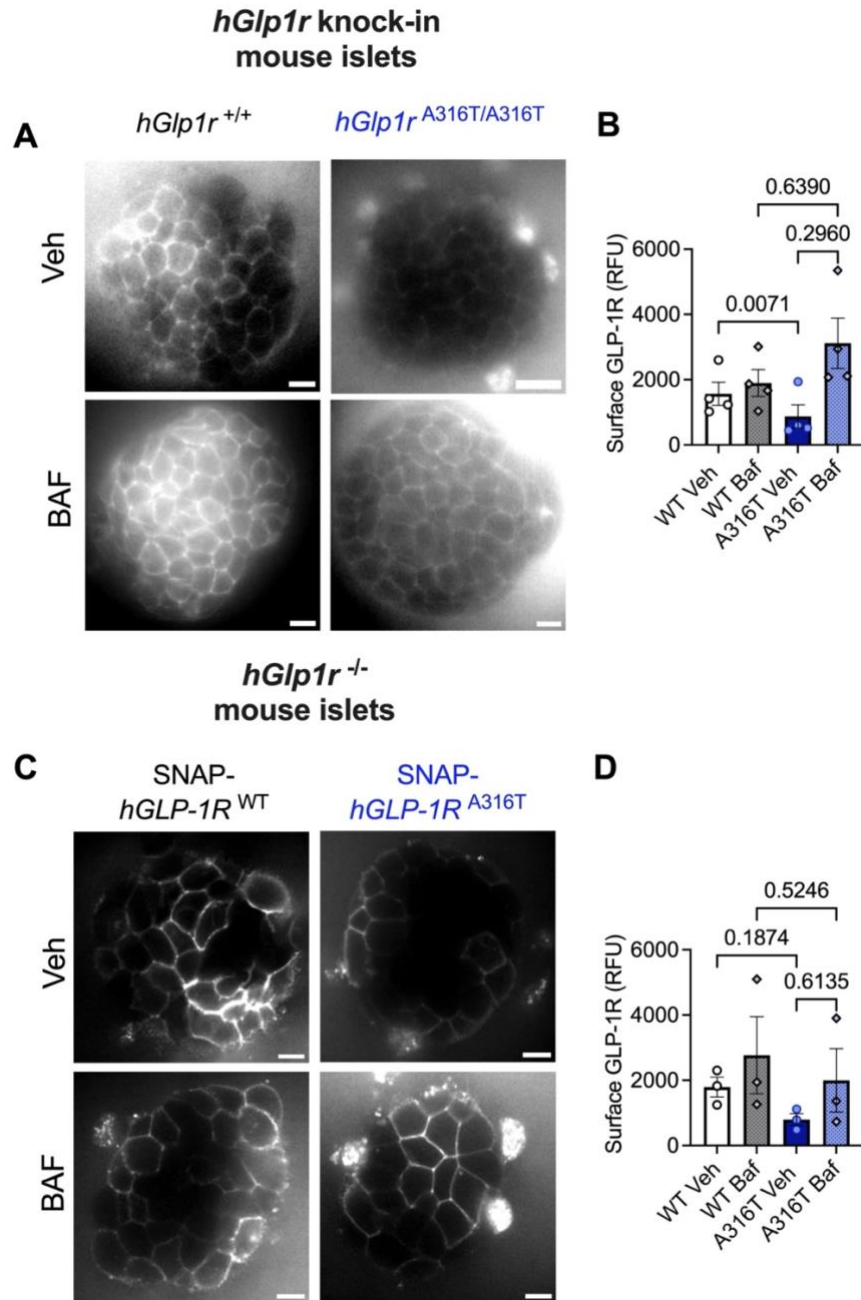


Figure 6.3 The A316T variant leads to enhanced basal lysosomal trafficking of the endogenous and heterogenous receptor in mouse islets. **A)** Representative fluorescence images of islets from *hGlp1r*^{A316T/A316T} or *hGlp1r*^{+/+} wild type controls, pre-treated or not for 2 hours with 400 μ M bafilomycin A1 (BAF), and **(B)** corresponding fluorescence levels from labelling with exendin-9-TMR. **C)** Representative fluorescence images of islets *hGlp1r*^{-/-} mouse islets expressing pAV-SNAP-hGLP-1R^{WT} or A316T, pretreated as (A), followed by labelling with SNAP-Surface Alexa Fluor 647 for 10 minutes, and **(D)** corresponding fluorescence levels.

Fluorescence from exendin-9-TMR or SNAP-Surface Alexa Fluor 647 is shown (white), and bars indicate 50 μm . Comparisons of A316T vs WT were made with one-way analysis of variance (ANOVA) with Sidak's post-hoc tests. Data are presented as means $\pm$ SEM.

6.2.4 The A316T variant exhibits altered agonist-induced trafficking profiles in murine islets

To corroborate the A316T specific agonist-induced internalisation profiles *in vitro*, I investigated the impact of the A316T mutation on GLP-1R agonist-induced internalisation profiles in *hGlp1r*^{-/-} mouse islets transduced with SNAP-hGLP-1R^{WT} or A316T adenoviruses, corresponding to the transient exogenous expression of these receptors. For this I used SNAP surface probes to label and visualise the surface receptor in these islets in the presence and absence of various GLP-1RAs (GLP-1, exendin-4, exendin-F1, and exendin-D3). As shown in Figure 6.4A, under basal conditions, the surface expression of the A316T receptor was significantly reduced compared to the WT receptor, consistent with previous findings. Moreover, with regards to the agonist-induced conditions, as expected treatment with these agonists generally reduced cell membrane localisation whilst increasing endosomal localisation of both WT and A316T GLP-1R (Figure 6.4A). This suggests that there is no visible change in agonist-induced endocytosis levels compared to the WT receptor. To evaluate the internalisation of both receptors, I quantified receptor endocytosis following treatment with exendin-F1. Exendin-F1 was selected for this analysis because it exhibits reduced internalisation compared to other agonists, allowing us to quantify receptor expression at the cell surface post-labelling, and thus a more detailed investigation of the internalisation process. Figure 6.4B and C illustrate that the A316T receptor exhibited similar cell surface expression levels after exendin-F1 treatment compared to the WT receptor ($p=0.4745$), yet

significantly less internalisation when compared to their respective vehicles ($p=0.0183$). A trend for reduced exendin-F1 internalisation was also observed in INS-1 832/3 cell lines before, as seen in Chapter 4, but it did not reach statistical significance there.

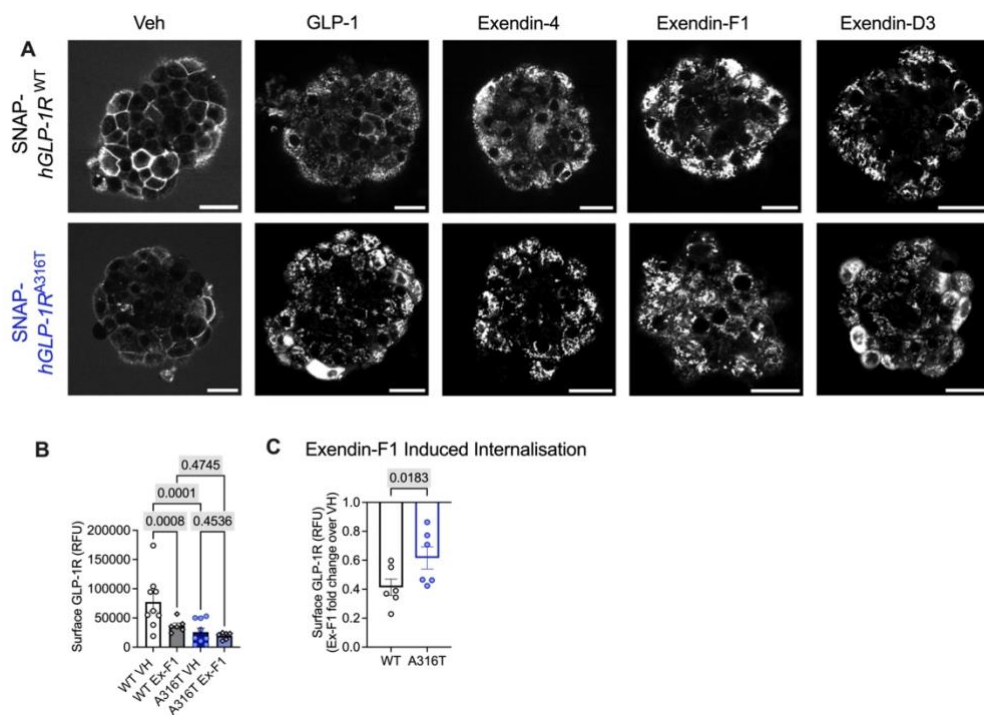


Figure 6.4 The A316T GLP-1R exhibits enhanced basal endocytosis and altered agonist-induced internalisation in mouse islets. **A)** Representative images of *hGlp1r*^{-/-} mouse islets transduced with pAV-SNAP-hGLP-1R^{WT} or -^{A316T} mutant receptor, labelled with SNAP-surface probe and treated with 100nM GLP-1, exendin-4, exendin-F1, exendin-D3 or vehicle (Veh) for 10 minutes. Scale bars are 20 μ m. Fluorescence for SNAP-hGLP-1R from SNAP-Surface probe is shown (white). **B)** Quantification of SNAP-hGLP-1R surface expression in Veh and exendin-F1 treated conditions and **C)** Exendin-F1 induced internalisation calculated as fold reduction in surface expression relative to vehicle. Comparisons of A316T vs WT were made with paired t-tests or one-way analysis of variance (ANOVA) with Sidak's post-hoc tests. Data are presented as means $\pm$ SEM.

6.2.5 The A316T variant exhibits agonist specific phenotypes of cAMP production in murine islets

To elucidate the specific impact of the A316T variant on GLP-1R downstream signalling pathways such as cAMP production, I utilised live-cell fluorescent microscopy with the genetically encoded cAMP biosensor cADDiS. Isolated islets from wild-type (*hGlp1r*^{+/+}) and A316T mutant (*hGlp1r*^{A316T/A316T}) mice were transduced with cADDiS and allowed to express the biosensor for 24 hours prior to experiments. Fluorescence changes were continuously recorded using live-cell microscopy to monitor real-time cAMP dynamics. After establishing baseline fluorescence for 2 minutes, islets were stimulated with a single 100 nM concentration of GLP-1RAs exendin-4 or exendin-F1 for 5 minutes. Finally, islets were treated with IBMX and forskolin, a phosphodiesterase inhibitor and known activator of adenylate cyclase respectively, to validate assay sensitivity and ensure it could accurately detect changes in cAMP levels. Prior to agonist stimulation, I measured baseline cADDiS fluorescence intensity to determine any inherent differences between WT and A316T islets. I observed that for A316T islets average baseline cAMP fluorescence levels were not significantly different compared to WT islets (Figure 6.5A top left panel, $p=0.430$) yet considering the reduced cell surface expression of this receptor, this value becomes significant (Figure 6.5A, lower left panel, $p=0.0461$).

Upon stimulation with exendin-4, A316T islets displayed less pronounced rises in cAMP levels compared to WT islets over a period of 5 minutes (Figure 6.5B), however AUCs were not significantly different as seen in Figure 6.5B ($p=0.9582$). On the other hand, A316T islets exhibited increased cAMP responses to exendin-F1 relative to wild-type islets over the 5-minute period (Figure 6.5C). The AUCs depicted in Figure 6.5D show a non-significant

increase in cAMP levels compared to WT islets following exendin-F1 stimulation ($p=0.2614$). This difference becomes significant when calculated as A316T fold changes to WT responses (Figure 6.5E). This live-cell imaging data underscores the significant impact of the endogenously expressed A316T variant on GLP-1RA induced cAMP signalling.

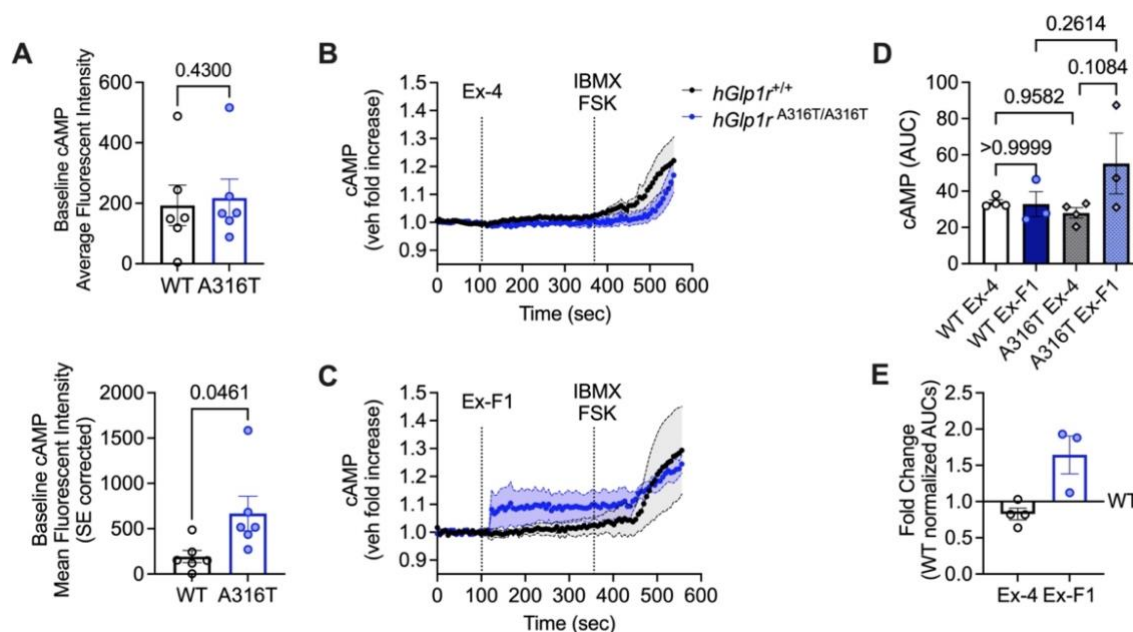


Figure 6.5 The *hGlp1r*^{A316T/A316T} mouse islets exhibit altered basal and agonist-induced downstream cAMP signalling. **A)** Average baseline cADDis cAMP sensor signal during the first 2 minutes prior to agonist addition, raw and surface expression corrected. **B and C)** cADDis cAMP sensor signal over time in response to addition of 100 nM exendin-4 (**B**) and exendin-F1 (**C**), followed by 100 μ M IBMX and forskolin at the indicated times in chow fed *hGlp1r*^{A316T/A316T} versus wild-type control islets. **(D and E)** Baseline normalised AUC values calculated for the treatment period (**D**) and wild-type normalised AUCs (**E**); $n=4$ per genotype. Comparisons of A316T versus WT controls islets were made with paired t-tests or one-way analysis of variance (ANOVA) with Sidak's post-hoc tests. Data are presented as means $\pm$ SEM.

6.2.6 The A316T variant exhibits agonist-specific intracellular calcium phenotypes in murine islets

Calcium signalling plays a crucial role in the regulation of various cellular processes, including hormone secretion, cell growth and differentiation. In pancreatic β -cells, calcium influx is a key event in the cascade leading to GSIS, which is essential for maintaining glucose homeostasis. Moreover, results from the *in vitro* experiments, described in section 4.2.11, showed that the A316T variant enhances GSIS independent of agonist stimulation in human β -cells, and previous studies found a GoF calcium mobilisation phenotype for this mutant (527). It is well established that enhanced GSIS is closely associated with elevated intracellular calcium mobilisation in pancreatic β -cells. Building upon this foundation, this study aimed to assess the impact of the A316T GLP-1R variant on calcium signalling dynamics using the fluorescent calcium indicator Cal520-AM as described in subsection 2.3.4. Baseline fluorescence intensities were initially recorded for 2 minutes to ensure that there were no significant differences between WT and A316T islets prior to agonist stimulation. Adenovirally transduced islets were then treated with 100 nM GLP-1RAs (GLP-1, exendin-4, exendin-F1, semaglutide and tirzepatide), followed by recording of calcium transients for a further 5 minutes and a subsequent elevation of glucose concentration to 11 mM from the initial 6 mM glucose.

A detailed comparison of Ca^{2+} mobilisation responses between WT and A316T expressing islets revealed modest differences in calcium transients at both 6 mM and 11 mM glucose concentrations. At 6 mM glucose, the endogenous agonist GLP-1, exendin-4, semaglutide and tirzepatide showed no significant differences in Ca^{2+} mobilisation in A316T islets compared

to WT controls (Figure 6.6A, B, C and D). Yet, Ca^{2+} mobilisation was significantly enhanced in the A316T islets in response to exendin-F1 at this glucose concentration. A316T islets also reached maximal responses faster than WT islets when stimulated with this agonist (Figure 6.6D). On the other hand, at 11 mM glucose, modest increases in Ca^{2+} mobilisation responses were observed upon stimulation of the A316T islets with GLP-1 and tirzepatide compared to WT islets (Figure 6.6A and E). Interestingly, despite the A316T exhibiting a higher total Ca^{2+} mobilisation response to tirzepatide, they exhibit a delay in reaching peak fluorescence compared to the WT islets (Figure 6.6E). Contrary to this, exendin-4-stimulated Ca^{2+} mobilisation responses were modestly attenuated along with the increase in glucose concentration, exhibiting dampened and irregular oscillations throughout the stimulation period (Figure 6.6B).

Lastly, I normalised AUC Ca^{2+} responses in the A316T group for all agonists at both glucose concentrations to the WT response by calculating the difference. This approach ensured that subtle differences in agonist-induced A316T responses could be more easily detected. Normalising the Ca^{2+} responses to WT highlighted several key insights, such as a significant increase in responses to exendin-F1 at 6 mM glucose with no changes in the response to other agonists relative to the WT (Figure 6.6F). I also observed an increase in Ca^{2+} signals upon stimulation with GLP-1 and tirzepatide, yet a reduction in response to exendin-4 stimulation, at 11mM glucose (Figure 6.6G).

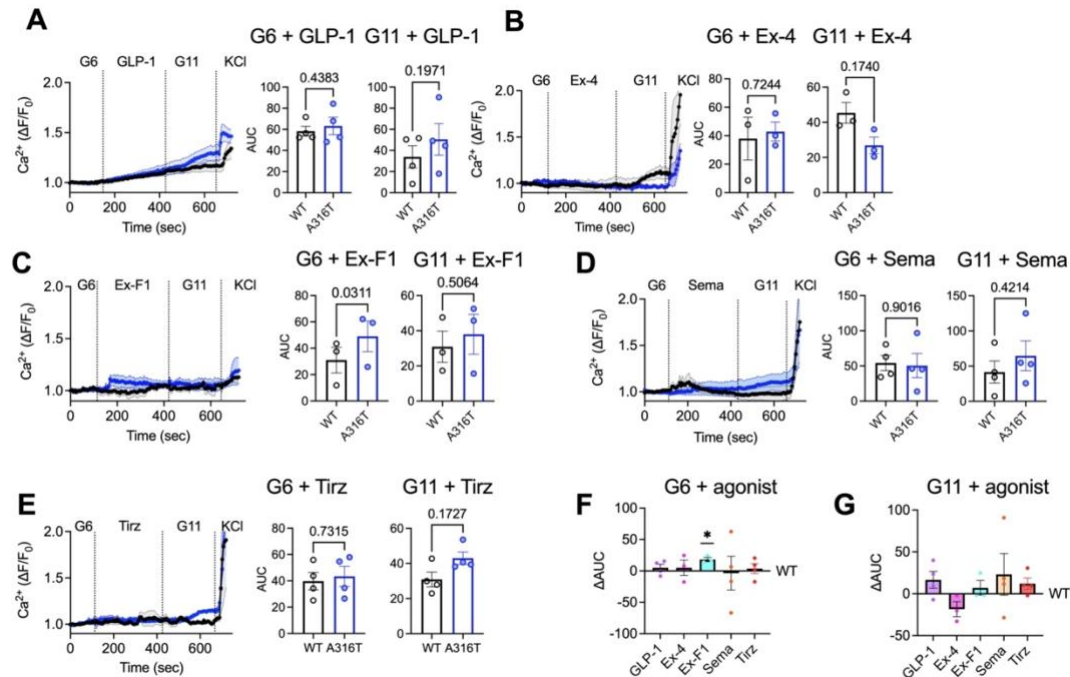


Figure 6.6 The A316T variant leads to altered basal and agonist-induced downstream calcium signalling responses. Calcium signalling measured by Cal520^{AM} biosensor fluorescence signal in *hGlp1r*^{-/-} mouse islets transduced with SNAP-hGLP-1R^{WT} or A316T adenoviruses post agonist addition. **A-E**) Calcium traces and corresponding AUCs for the indicated treatments at a concentration of 100 nM in the islets mentioned above, GLP-1 (**A**), exendin-4 (Ex-4) (**B**), exendin-F1 (Ex-F1) (**C**), semaglutide (Sema) (**D**), tirzepatide (**E**). **F-G**) Comparison of variant-specific agonist responses by calculating difference of A316T and WT AUCs at 6 mM glucose (G6) (**F**) and 11 mM glucose (G11) (**G**). Comparisons of A316T *versus* WT control islets were made with paired t-tests or two-way analysis of variance (ANOVA) with Sidak's post-hoc tests. **p* < 0.05, ***p* < 0.01, and ****p* < 0.0001; ns=not significant. Data are presented as means ± 95% CIs.

6.2.7 The A316T variant enhances GSIS in murine and human islets

Following on from previous findings on cAMP signalling in islets isolated from gene-edited knock-in *hGlp1r*^{+/+} / *hGlp1r*^{A316T/A316T} mice and calcium mobilisation in *hGlp1r*^{-/-} mice islets transiently expressing SNAP-GLP-1R^{WT} or A316T, this subchapter explores the downstream effects of this mutation on insulin secretion. As observed previously, the GLP-1R plays a pivotal role in glucose homeostasis, and its action including trafficking and signalling can be

altered by mutations such as the A316T, which affect both calcium mobilisation and cAMP signalling. This analysis involves both acute and overnight stimulation under different dietary conditions including chow and HFHSD, as well as treatment with semaglutide, a clinically leading GLP-1RA, in islet from the gene edited knock-in mouse model. Additional similar analyses were performed in adenovirally transduced murine *hGlp1r*^{-/-} islets and primary human islets transduced with the SNAP-hGLP-1R^{WT} or A316T variant.

*Investigating GSIS in *hGlp1r*^{A316T/A316T} and *hGlp1r*^{+/+} mouse islets*

In line with the increased basal plasma insulin levels previously reported for both acute and prolonged GLP-1RA treatment in chow and HFHSD-fed *hGlp1r*^{A316T/A316T} mice compared to *hGlp1r*^{+/+} littermate controls, Figures 6.7A and B present insulin secretion results for acute and prolonged stimulation with 11 mM glucose (basal response), exendin-4 and exendin-F1 in chow-fed mice. A significant increase in basal GSIS was observed for *hGlp1r*^{A316T/A316T} islets compared to *hGlp1r*^{+/+} controls (p=0.0376), whilst no difference was observed in response to all agonists under acute conditions (Figure 6.7A). However, Figure 6.7B highlights a non-significant increase in basal GSIS and no significant differences in agonist-induced GSIS between the *hGlp1r*^{A316T/A316T} and *hGlp1r*^{+/+} control islets in the overnight group. The right panels of Figure 6.7A and B highlight that this basal increase in GSIS becomes more pronounced upon correction of values for reduced cell surface expression of the mutant receptor. To eliminate inherent differences in baseline insulin secretion values and assess the insulin secretion efficacy of the different agonists in the presence of the A316T mutation, values in Figure 6.7A and B were normalised (folded) to 11 mM glucose levels, as shown in Figure 6.7C.

No significant differences in insulin secretion were observed between exendin-4 and exendin-F1 responses under both acute and overnight stimulation in *hGlp1r^{A316T/A316T}* islets, while, consistent with previous studies, results showed enhanced insulin secretion upon stimulation with exendin-F1 compared to exendin-4 in the *hGlp1r^{+/+}* overnight group, when normalised to 11 mM glucose levels. Figure 6.7C also highlights a significant decrease in insulin secretion responses to both agonists relative to 11 mM glucose alone between the *hGlp1r^{+/+}* group and the *hGlp1r^{A316T/A316T}* group, which is likely due to inherent basal insulin secretion exhibited by this group. Finally, the reduction in insulin secretion response between prolonged and acute time-points was more pronounced in the *hGlp1r^{+/+}* for both agonists compared to the *hGlp1r^{A316T/A316T}* (Figure 6.7D, $p=0.1208$ for exendin-4 and $p=0.0843$ for exendin-F1).

In parallel to this, Figures 6.7E and F display insulin secretion data for HFHSD-fed gene-edited mice under acute and overnight conditions, respectively. Acute stimulation results (Figure 6.7E) showed no significant differences between *hGlp1r^{+/+}* and *hGlp1r^{A316T/A316T}* in terms of basal or agonist-induced insulin secretion acutely (Figure 6.7E). In contrast, overnight stimulation showed attenuated insulin secretion responses for all conditions studied including basal and agonist-induced responses in the *hGlp1r^{A316T/A316T}* islets. As with the chow-fed mouse islets, correction for surface expression for the mutant-expressing islets enhanced insulin secretion responses observed, and this effect was more pronounced under acute stimulation compared to prolonged stimulation (Figure 6.7E and F, right panels). Moreover, normalising to baseline 11 mM glucose did not reveal any significant differences between *hGlp1r^{+/+}* and *hGlp1r^{A316T/A316T}* islets for agonist-induced responses under acute or overnight conditions. Despite the difference between overnight and acute responses being unchanged,

exendin-F1 stimulation elicited significantly enhanced overnight insulin secretion responses compared to acute stimulations, with the opposing results seen for exendin-4 stimulation.

Finally, I assessed the effect of the long-acting agonist semaglutide over a 24-hour treatment period, as shown in figures 6.7I and J. As before, a modest non-significant increase in basal insulin secretion was observed for the *hGlp1r*^{A316T/A316T} islets compared to control wild-type islets, and this difference became significant upon receptor cell surface expression correction (p=0.0078). Semaglutide treatment did not evoke any marked alterations in insulin secretion (Figure 6.7I and J). Yet, normalisation of agonist-induced insulin secretion responses to those evoked by 11 mM glucose reveal that while treatment with semaglutide enhances insulin secretion in *hGlp1r*^{+/+} islets, it does not promote any change in insulin secretion in *hGlp1r*^{A316T/A316T} islets relative to 11 mM glucose stimulation alone (Figure 6.7J, p=0.061).

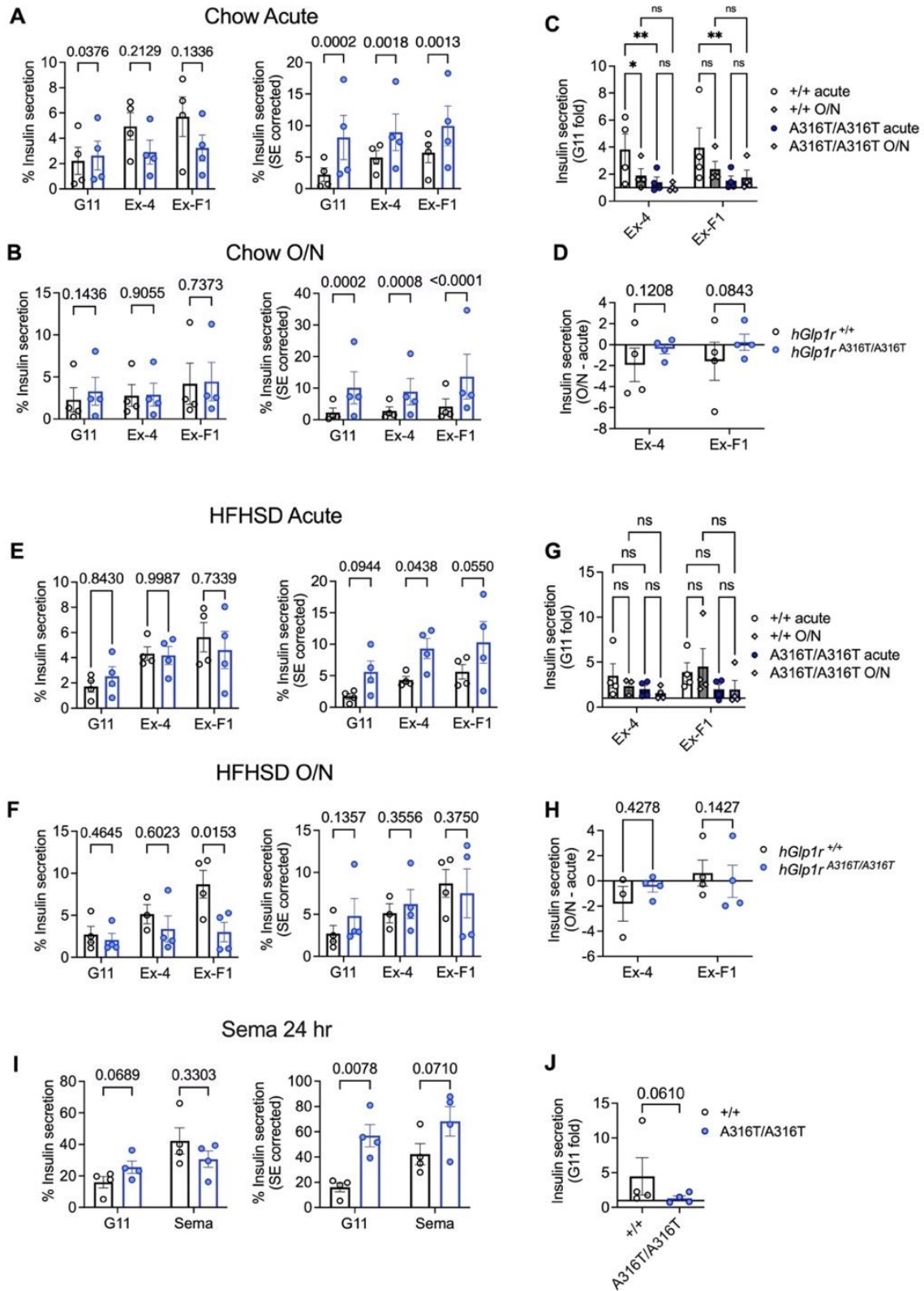


Figure 6.7 The *hGlp1r*^{A316T/A316T} mouse islets exhibit enhanced glucose stimulated insulin secretion under chow and HFHSD conditions. A-B) Effect of the A316T mutation on insulin secretion in the presence of 11 mM glucose (G11) only, or with 10 0nM exendin-4 or exendin-F1 (Ex-F1) treatment both acutely (30 minutes) (**A**) and over longer period (16/18 hours) (**B**) in chow-fed mouse islets and in HFHSD-fed mouse islets (**E-F**); **C and G**) Normalised % insulin secretion values demonstrating the effect of Ex-4 and Ex-F1 versus G11 alone, calculated from data from (**A and B**) and (**E and F**), and the difference between long-term and acute agonist responses, (FC LT- FC ST) from data in (**A and B**) and (**E and F**). (**I**) Effect of the A316T mutation on insulin secretion in the presence of high glucose (11 mM glucose) only or with 100 nM semaglutide treatment for 24 hours and normalized to G11 treatment alone (**J**). Comparisons of A316T *versus* WT controls islets were made with paired t-tests or two-way analysis of variance (ANOVA) with Sidak's post-hoc tests. *p < 0.05, **p < 0.01, and ***p < 0.0001; ns=not significant. Data are presented as means ± SEM.

*Investigating GSIS in *hGlp1r*^{-/-} mice islets transiently expressing SNAP-hGLP-1R^{WT} or *A316T**

As observed in experiments assessing insulin secretion in knock-in mouse islets, the presence of the A316T variant enhances GSIS yet reduces acute responses to GLP-1R agonists like exendin-4. In a similar manner, I aimed to determine if the inherent insulintropic effects of the A316T variant would be maintained when the receptor variant is exogenously rather than endogenously expressed.

This subsection focuses on GSIS in islets from *hGlp1r*^{-/-} mice transduced with adenoviruses expressing either WT or A316T receptors. Islets were isolated from *hGlp1r*^{-/-} mice and transduced with pAV SNAP-hGLP-1R^{WT} or *A316T* for 24 hours prior to conducting GSIS assays to compare the functional impact of the receptors on insulin release in this model. Contrary to previous results, both WT and A316T-expressing islets showed similar basal insulin secretion levels, yet consistent with previous results, A316T expressing islets exhibited significantly higher insulin secretion upon correction for cell surface receptor expression levels (Figure 6.8A, p=0.0012). Upon stimulation with the GLP-1RAs exendin-4 and exendin-F1, semaglutide, and tirzepatide, A316T-expressing islets exhibited unchanged responses to exendin-4, modestly decreased response to semaglutide, significantly decreased insulin secretion with exendin-F1

and a tendency for increased insulin secretion in response to tirzepatide stimulation (Figure 6.8B and C, left panels). As differences in receptor cell surface expression could affect efficacy of agonist responses, I corrected A316T insulin secretion responses to its cell surface expression levels, which resulted in an overall increase in agonist-stimulated insulin responses in A316T compared to WT GLP-1R (Figures 6.8B and C right panels). Figures 6.8D and E show the fold change in insulin secretion (stimulated *versus* basal) for both WT- and A316T-expressing islets. The fold change was modestly reduced for A316T in response to exendin-4, exendin-F1, and semaglutide, but increased in response to tirzepatide. This indicates that the A316T mutation exhibits distinct effects on the activity of the different agonists, and this may be due to underlying biased-agonism characteristics accentuated by the mutation, leading to a more pronounced insulintropic response in A316T-expressing islets.

Investigating GSIS in primary human islets transiently expressing SNAP-hGLP-1R^{WT} or A316T

GSIS experiments in primary human islets were essential to validate the findings observed in both the gene-edited endogenous *hGlp1r^{A316T/A316T}* islets and exogenous A316T-expressing islets. Primary human islets provide a more clinically and physiologically relevant model, ensuring that the observed effects of the A316T variant on insulin secretion translate to human physiology. This also gave me an opportunity to validate insulin secretion findings obtained in the EndoC- β H3 human pancreatic β -cell line, in a more complex human tissue context comprising multiple endocrine cell types. Figures 6.8F and G present the GSIS response in primary human islets transduced with adenoviruses expressing GLP-1R A316T or WT. Similar to knock-in mouse islets, there was a non-significant increase in basal insulin secretion ($p=0.0671$), which became significant upon receptor surface expression correction ($p=0.018$) in A316T compared to WT GLP-1R (Figure 6.8F). Conversely, there was no significant difference

in insulin secretion in these islets in response to the GLP-1RA exendin-4 and marked reductions in response to the biased exendin analogues exendin-F1 and exendin-D3. These were less pronounced upon receptor cell surface expression correction (Figure 6.8G).

Insulin secretion differences observed between the genotypes were further highlighted in Figure 6.9H, whereby fold change analysis in insulin secretion (stimulated *versus* basal) for both WT and A316T expressing islets revealed significantly lower fold change for A316T upon stimulation with exendin-F1 and exendin-D3, as well as a decrease in response to exendin-4. This indicates attenuated insulin secretion upon agonist stimulation due to the A316T mutation, most likely due to the inherent robust enhancement in insulin secretion in response to glucose stimulation alone.

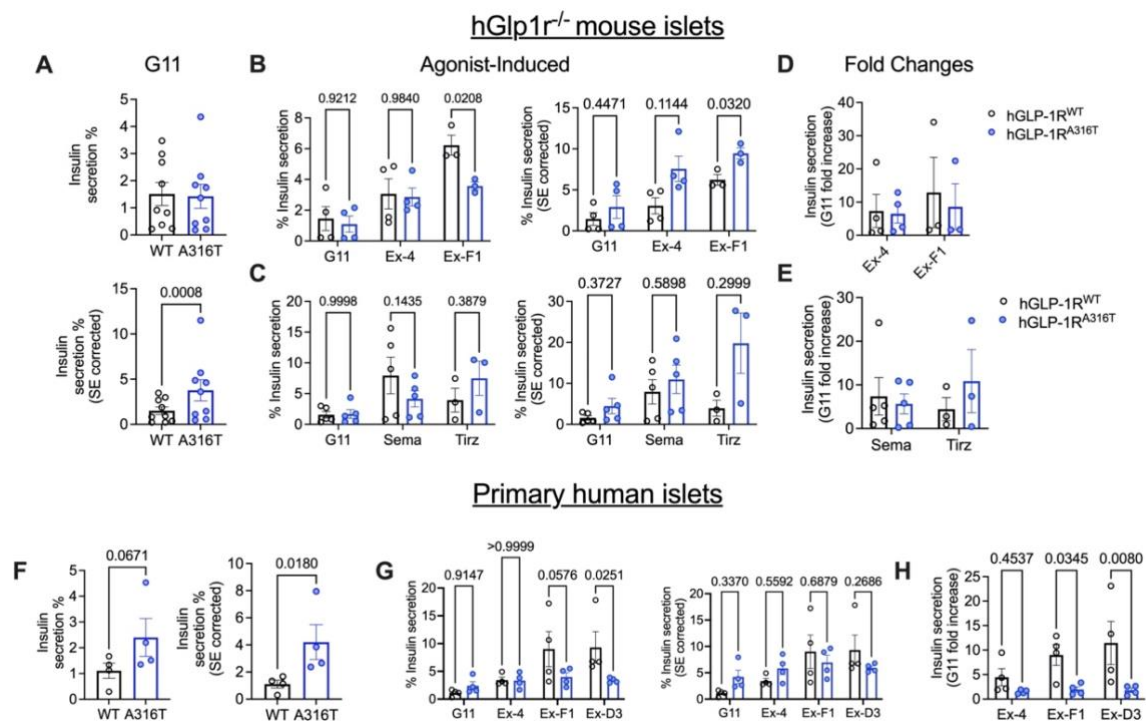


Figure 6.8 The A316T variant leads to enhanced basal glucose stimulated insulin secretion in human and mouse islets **A-C)** Effect of the A316T mutation on insulin secretion in the presence of 11mM glucose (G11) only (**A**), or with short-acting GLP-1RAs (**B**) 100 nM exendin-4 (Ex-4) or exendin-Phe (Ex-F1), or alternatively with (**C**) long-acting clinical agonists semaglutide (Sema) or tirzepatide in *hGlp1r*^{-/-} mouse islets expressing pAV-SNAP-hGLP-1R WT or -A316T mutant. **D and E)** Normalized % insulin secretion values demonstrating the effect of Ex-4 and Ex-F1 versus G11 alone or the effect of semaglutide and tirzepatide versus G11 alone, calculated from data from **B** and **C**, respectively. **F-G)** Effect of the A316T mutation on insulin secretion in the presence of G11 only (**F**), or with GLP-1RAs (**G**) 100 nM Ex-4, Ex-F1, and Ex-D3. **(H)** Normalized % insulin secretion values demonstrating the effect of Ex-4, Ex-F1, and Ex-D3 versus G11 alone. Comparisons of A316T *versus* WT controls islets were made with paired t-tests or two-way analysis of variance (ANOVA) with Sidak's post-hoc tests. *p < 0.05, **p < 0.01, and ***p < 0.0001; ns=not significant. Data are presented as means ± SEM.

6.2.8 Investigating islet cyto-architecture in *hGlp1r*^{A316T/A316T} and *hGlp1r*^{+/+} mouse islets

In this study, I examined the islet cell composition in purified *hGlp1r*^{A316T/A316T} and *hGlp1r*^{+/+} control mice islets. This analysis was performed using isolated islets followed by immunofluorescence staining to visualise insulin and glucagon within these islets, which are markers for β- and α-cells, respectively. The stained sections were then analysed to determine islet diameter, β- and α-cell numbers, and the proportion of β- and α-cells within the islets. The rationale for this investigation stems from previous observations in *hGlp1r*^{A316T/A316T} frozen pancreatic tissue sections of marked differences in α-cell mass specifically under diet-induced metabolic stress conditions. In addition to this, I aimed to understand underlying molecular mechanisms of significant improvements in β-cell function and GSIS in isolated *hGlp1r*^{A316T/A316T} islets.

Islet cell composition under chow diet

Figure 6.9A shows representative immunofluorescence images of islets from *hGlp1r*^{+/+} and *hGlp1r*^{A316T/A316T} mice on a chow diet. The images illustrate the distribution of insulin and

glucagon within the islets. Under a chow diet, there was no significant difference in overall islet diameter between *hGlp1r*^{+/+} and *hGlp1r*^{A316T/A316T} islets (Figure 6.9B). Conversely, *hGlp1r*^{A316T/A316T} islets exhibited a significant increase in β -cell numbers by about 2-fold ($p=0.0480$), whilst there was no significant difference observed in α -cell numbers compared to *hGlp1r*^{+/+} control islets (Figure 6.9C). Although, I observed a trend for increased percentage of β -cells in the *hGlp1r*^{A316T/A316T} islets, this was not significant ($p=0.2122$), and the percentage of α -cells reflected no change compared to *hGlp1r*^{+/+} islets (Figure 6.9D).

Islet cell composition under HFHSD

Figure 6.9F presents immunofluorescence images of islets from *hGlp1r*^{+/+} control and *hGlp1r*^{A316T/A316T} mutant mice on a HFHSD. Consistent with the A316T-specific differences observed in pancreatic tissue sections, staining patterns were altered in the presence of the homologous A316T mutation. Unlike findings under the chow diet, I observed a significant increase in islet diameter in the *hGlp1r*^{A316T/A316T} compared to the *hGlp1r*^{+/+} control islets (Figure 6.9G, $p=0.0304$). Furthermore, under HFHSD, the increase in β -cell numbers in *hGlp1r*^{A316T/A316T} islets was no longer present, while there was a significant increase in α -cell numbers instead (Figure 6.9H, $p=0.0473$). Congruently, the percentage of α -cells was also significantly increased ($p=0.0276$), while the percentage of β -cells was significantly reduced ($p=0.0233$) in *hGlp1r*^{A316T/A316T} compared to *hGlp1r*^{+/+} islets under HFHSD (Figure 6.9I). The increase in α -cell numbers and their proportion under HFHSD in *hGlp1r*^{A316T/A316T} islets suggests a shift in islet cell composition, consistent with findings in pancreatic tissue sections, in response to metabolic stress, potentially leading to dysregulated glucagon secretion.

Of note, differences in islet diameter between *hGlp1r*^{+/+} and *hGlp1r*^{A316T/A316T} mutant mice should be interpreted cautiously, as the islet isolation process may bias recovery towards islets from different regions of the pancreas. For instance, larger islets are more abundant in the tail, and if the digestion process differentially favours tail *versus* head/body regions between genotypes, this could skew diameter measurements independently of genotype-specific effects.

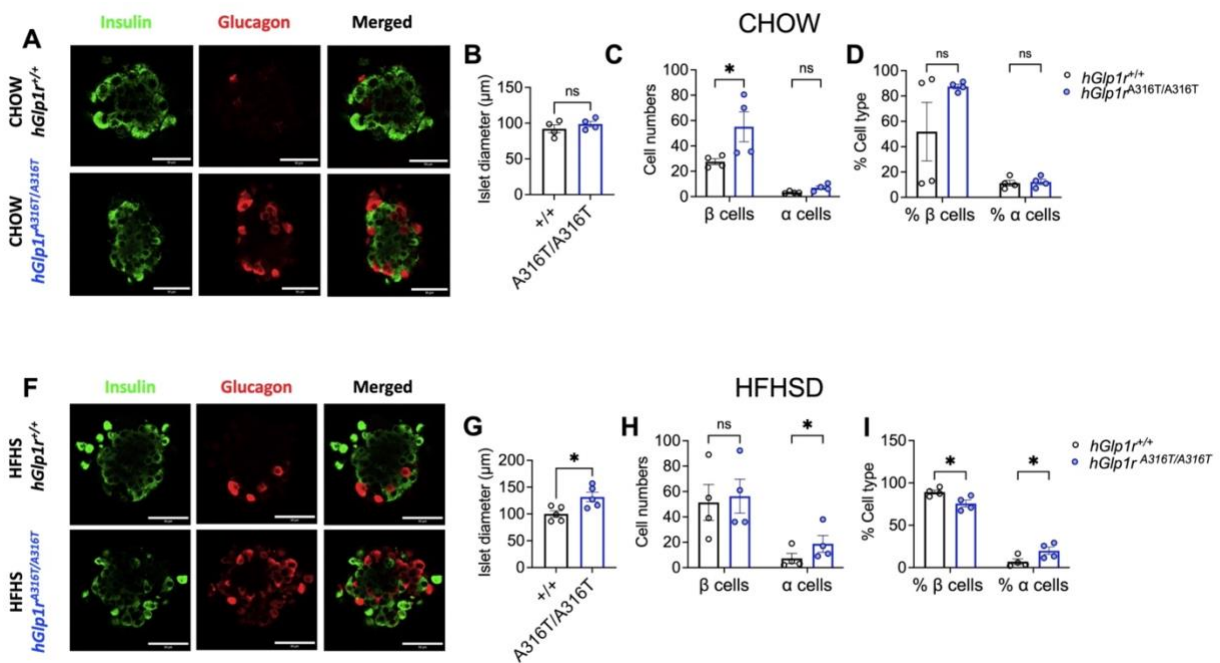


Figure 6.9 Islet morphology and cell composition is altered in *hGlp1r*^{A316T/A316T} mice under HFHSD conditions. A-B) Morphology of pancreatic islets isolated from *hGlp1r*^{A316T/A316T} and wild-type littermates from mice fed with either standard (A) chow diet or (B) HFHSD. Immunofluorescence for insulin (green) and glucagon (red) are shown, and bars indicate 50 μm. Corresponding islet measurements including islet diameters (B and G), cell numbers (C and H) and % cell type (D and I).

6.3 Discussion

To connect the physiological relevance observed in vivo with the mechanistic insights gained in vitro, I validated my findings on the A316T variant, including its intrinsic GoF phenotype,

using ex vivo murine islet models from endogenous human GLP-1R-expressing knock-in mice, as well as *hGlp1r*^{-/-} and primary human islets exogenously overexpressing the WT or A316T GLP-1R via adenoviruses. This method allows for precise control of experimental conditions, free from systemic influences such as neural input, non-pancreatic hormone signalling, and interactions with other organs that may be altering glucose metabolism and pharmacological responses. By utilising this approach, I can investigate specific cellular and molecular pathways to provide a comprehensive understanding of how the A316T variant alters islet function linking molecular and cellular mechanisms with systemic physiological outcomes observed in vivo.

6.3.1 Investigating A316T induced alterations cell surface expression and trafficking in ex vivo murine and human mouse islets

Firstly, I aimed to validate the reduced cell surface expression of the A316T GLP-1R in islets expressing the endogenous A316T from *hGlp1r*^{A316T/A316T} mice and the exogenous SNAP-GLP-1R A316T in *hGlp1r*^{-/-} and primary human islets. I confirmed that the A316T GLP-1R exhibits significantly reduced surface expression in all models studied. Using exendin-9-TMR fluorescent labelling, I showed that A316T GLP-1R exhibited significantly reduced cell surface expression compared to wild-type controls, in islets from chow- and HFHSD-fed mice. This reduction was more pronounced under HFHSD, indicating a diet-induced exacerbation of receptor expression deficiency in the mutant mice, probably as a result of further exaggerated basal trafficking mechanisms. Employing SNAP-tag labelling in *hGlp1r*^{-/-} and primary human islets exogenously expressing the SNAP tagged receptors, revealed results mirroring those seen with endogenous expression.

In a similar vein, inhibition of lysosomal degradation partially restored surface expression of the A316T receptor in all three models studied, corroborating previous studies showing increased basal lysosomal targeting by this mutant as the reason behind its cell surface expression defect.

Lastly, I observed altered agonist-induced trafficking profiles. Mirroring its increased basal lysosomal degradation profile, the A316T receptor also showed increased internalisation, similar to that induced by agonists, indicating a higher basal endocytosis rate compared to the WT receptor. Agonist treatment increased endosomal localisation for both receptor types, but the A316T receptor internalisation was significantly reduced with exendin-F1 compared to the WT receptor, aligning with a tendency for reduced internalisation with exendin-F1 for the A316T in INS-1 832/3 cells. Therefore, it seems that as a result of exendin-F1 induced conformational arrangements in the A316T receptor structure, this agonist exhibits a more pronounced exendin-F1 effect maintaining the receptor on the cell surface for longer periods of time.

A limitation of the agonist-induced trafficking study performed is that it was not quantifiable for the other agonists due to the increased basal endocytosis rate of the A316T GLP-1R which resulted in lack of measurable cell surface expression. Hence, to investigate internalisation more accurately, as well as other trafficking pathways such as recycling, islets could be treated with acetic acid buffer wash after fluorescent agonist exposure which would allow to quantify internalised versus surface receptors (545). Alternatively, further studies to investigate basal trafficking could be performed, such as treating islets with inhibitors like dynasore to block endocytosis or brefeldin A to interfere with recycling, which will aid in the identification of specific pathways affected by the A316T mutation.

6.3.2 Investigating A316T induced alterations in cAMP production and calcium mobilisation in *ex vivo* murine and human islets

In assessing cAMP production and downstream signalling effects of the A316T mutation in *hGlp1r*^{A316T/A316T} islets, live-cell fluorescent microscopy with the genetically encoded cADDiS cAMP biosensor monitoring real-time cAMP dynamics revealed that average baseline cAMP production in *hGlp1r*^{A316T/A316T} resembled that in *hGlp1r*^{+/+} control islets. This could be explained by the significantly enhanced basal G_{i/o} recruitment regulating cAMP levels and preventing overstimulation. However, upon stimulation with exendin-4, A316T expressing islets showed a less pronounced increase in cAMP levels compared to wild-type islets. Conversely, exendin-F1 induced a rapid and pronounced increase in cAMP levels in the mutant islets, suggesting agonist-specific differences in cAMP signalling between the mutant and the WT receptors. These agonist-specific findings also align with previous *in vivo* IPGTT and *in vitro* human pancreatic β -cell insulin secretion studies.

Calcium signalling is another important readout of GLP-1R activation, which determines GSIS in pancreatic β -cells. Using the fluorescent calcium indicator Cal520-AM as in INS-1 832/3 cells, I assessed the impact of the A316T GLP-1R variant on calcium signalling in islet models in the presence and absence of agonist stimulation. Consistent with *in vitro* calcium signalling studies in INS-1 832/3, insulin secretion findings *in vivo*, and upregulated *Kcnj11* in *hGlp1r*^{A316T/A316T} islets, average baseline calcium levels were significantly higher in A316T-expressing *hGlp1r*^{-/-} islets compared to WT-expressing controls, indicating again an inherent GoF phenotype for this pathway. On the other hand, upon stimulation with various GLP-1RAs

at 6 mM glucose, only exendin-F1 significantly enhanced calcium mobilisation in A316T islets.

The enhanced calcium responses of the A316T variant correlated with a milder impairment in glucoregulation observed at prolonged time-points with this agonist *in vivo*, perhaps linked with reduced desensitisation tendencies affecting less the A316T variant which has a tendency for increased desensitisation. Further research is warranted to explore the effect of this mutation on downstream signalling pathway such as cAMP and calcium mobilisation in response to different glucose concentrations. As different glucose concentrations mimic various physiological states, from fasting (low glucose) to post-prandial (high glucose), aiding in the elucidation of metabolic-state specific responses using a range of glucose doses can uncover deeper mechanistic insights into how the A316T mutation affects GLP-1R signalling. The same occurs for GLP-1RA concentrations where higher doses might reveal compensatory mechanisms or saturation points, while lower doses could indicate sensitivity changes. In addition to this, we can map dose-response relationships, which can help identify the optimal therapeutic dose with maximum efficacy.

6.3.3 Investigating A316T-associated insulintropic effects in *ex vivo* murine and human islets

Insulin secretion assays were performed to evaluate if the A316T associated phenotypes translate to insulintropic effects in *ex vivo* mouse islets endogenously or exogenously expressing the mutant receptor, and in human islets.

In *hGlp1r*^{A316T/A316T} islets, basal GSIS was higher compared to wild-type controls, significantly under chow diet conditions and non-significantly under HFHSD conditions, consistent with

effects in basal cAMP and calcium levels. However, no significant differences in agonist-induced GSIS were observed between mutant and wild-type islets, both under acute and overnight stimulation. This suggests that while the A316T mutation enhances basal insulin secretion levels, it does not allow for increased GLP-1RA effects in potentiating insulin secretion. This is likely due to the partially active state of the A316T variant independent of any agonist activation or perhaps because the system is saturated and higher levels of secretion are not possible due to other rate limiting effects. Under HFHSD conditions, prolonged overnight stimulation revealed attenuated insulin secretion responses in *hGlp1r*^{A316T/A316T} islets with exendin-4 and exendin-F1. This attenuation might be attributed to the metabolic stress imposed by the HFHSD, which might exacerbate the effects of the A316T mutation over prolonged stimulations. In parallel to this, assessment of the long-acting GLP-1R agonist semaglutide over a 24-hour period indicated a modest increase in basal insulin secretion in *hGlp1r*^{A316T/A316T} islets but no potentiation of insulin secretion relative to glucose stimulation alone for this long-acting agonist. These findings further highlight the inherent enhanced insulinitropic effects of the A316T variant, overshadowing any agonist-induced effects, especially over longer stimulation periods.

In *hGlp1r*^{-/-} islets transduced with SNAP-hGLP-1R^{WT} or ^{A316T} receptors, A316T-expressing islets exhibited markedly higher basal insulin secretion levels, mirroring findings in islets expressing endogenous receptors. Agonist stimulation showed varied responses, with exendin-4 and semaglutide eliciting reduced responses, while tirzepatide showed a tendency for increased insulin secretion in A316T-expressing islets. These results suggest that the mutation might affect the efficacy of specific agonists in a context specific manner. There is a discrepancy between islet secretion and glucose responses to tirzepatide, as despite the

increased insulin secretion *ex vivo*, IPGTT results showed impaired glucose tolerance compared to wild-type controls. Tirzepatide may induce different signalling pathways in the presence of the A316T mutation, leading to enhanced insulin secretion without a proportionate increase in glucose tolerance due to its biased profile. Interestingly, this phenomenon can be compared to similar effects with exendin-P5, which also shows enhanced insulin secretion without corresponding improvements in glucose tolerance (385). This suggests that while activation of the human A316T GLP-1R by tirzepatide effectively stimulates β -cells to release insulin, other factors such as biased agonism or tissue-specific responses limit its ability to lower blood glucose levels effectively. Glucose uptake in various tissues (e.g. muscle, liver, adipose tissue) following administration of agonists or in the basal state can be assessed using techniques such as positron emission tomography (PET) scanning with glucose analogues, providing insights into how these tissues respond to insulin secretion stimulated by these agonists. Intra-peritoneal insulin tolerance tests (IPITTs) could have also been performed here in the *in vivo* model.

Lastly, experiments in primary human islets transduced with adenoviruses expressing the SNAP-tagged hGLP-1R^{WT} or A316T confirmed findings from murine models. A marked increase in basal insulin secretion was observed in A316T-expressing human islets. However, responses to GLP-1R agonists were attenuated, particularly for exendin-F1 and exendin-D3, indicating a robust enhancement in basal GSIS that may overshadow the effects of agonist stimulation. These results validate the positive metabolic impact of the A316T mutation on insulin secretion in a more clinically relevant model and highlight the complex interplay between receptor signalling, genetic mutations and biased agonism, providing valuable

insights for developed targeted therapies for metabolic disorders in the presence of GLP-1R mutations.

Another model that could be used to characterise basal and simulated insulin secretion, cAMP signalling, and calcium mobilisation involves the generation of induced pluripotent stem cell (iPSC)-derived islets genetically edited to express the A316T GLP-1R variant. These iPSC-derived islets would provide a robust and versatile model to study pancreatic β -cell function and impact of genetic mutations such as the A316T variant, whilst mimicking human physiology, and enabling detailed mechanistic and drug screening studies.

Employing real-time techniques to study insulin secretion in murine or primary human islet models expressing the A316T variant would also provide higher temporal and spatial resolution, enabling a deeper understanding of the molecular and cellular processes involved in insulin secretion. These techniques include dynamic perfusion systems and total internal reflection fluorescence (TIRF) microscopy which allow for real-time monitoring of insulin granule exocytosis, providing detailed kinetic data on insulin secretion responses as well as detailed insights into the final steps of insulin exocytosis in the presence of the A316T mutation.

6.3.4 Investigating A316T-associated changes in *ex vivo* islet cell composition

To elucidate alterations in islet cell composition in *hGlp1r*^{A316T/A316T} islets, I used immunofluorescence staining for insulin and glucagon, markers for β - and α -cells, respectively. I found an increase in β -cell numbers under a chow diet in *hGlp1r*^{A316T/A316T} islets correlating with improved basal insulin secretion in these islets. Thus, the A316T variant

enhances β -cell proliferation, contributing to enhanced basal insulin secretion and improved glucose homeostasis under normal dietary conditions. However, the lack of significant differences in β -cell numbers under HFHSD highlights the impact of metabolic stress on islet composition, suggesting that other compensatory pathways are involved in promoting the enhanced GSIS under metabolic stress conditions.

Remarkably, I observed a marked increase in α -cell numbers and proportion in *hGlp1r^{A316T/A316T}* islets under HFHSD conditions, aligning with findings in pancreatic tissue sections from the same mice, suggesting a shift towards α -cell predominance with the A316T variant under metabolic stress. One potential explanation is that enhanced GLP-1R activity in the *hGlp1r^{A316T/A316T}* mice indirectly alters systemic nutrient handling. The A316T variant increases GLP-1R signalling even in the absence of exogenous agonist, driving enhanced basal insulin secretion. This may skew hepatic nutrient sensing and glucose-lipid portioning, inadvertently reducing hepatic amino acid clearance. Consequently, circulating amino acids may remain elevated and stimulate α -cell proliferation and glucagon secretion via the liver- α -cell axis — a well-described compensatory loop in response to chronic hyperaminoacidaemia. While GLP-1R agonists are known to suppress glucagon secretion, under basal or untreated conditions, increased glucagon secretion may still occur and play a role in islet adaptation. In addition to these indirect systemic effects, it is also possible that altered paracrine dynamics within the islet contribute to this phenotype. Increased β -cell activity may modulate intra-islet signalling, either suppressing somatostatin-mediated α -cell restraint or altering local growth factor cues. Although α -cells express relatively low levels of GLP-1R, under stress conditions GLP-1R signalling could influence α -cell fate, possibly promoting expansion or altered differentiation. To test these hypothesis, future studies should assess Ki67 staining in

pancreatic tissue to directly quantify proliferative activity within the α -cell population. In parallel, measuring total amino acid levels in HFHSD mice in the fasted state could determine whether chronic amino acid elevation underlies the observed α -cell expansion. Interestingly, though, in *hGlp1r*^{A316T/A316T} islets, despite the significant α -cell expansion under metabolic stress, enhanced basal insulin secretion is still maintained. Several mechanisms may underlie this paradox. First, certain α -cell populations can secrete GLP-1 under hyperglycaemic conditions (226, 633, 634), and increased α -cell numbers may boost local GLP-1 availability, further enhancing β -cell insulin secretion through paracrine signalling.

Chapter 7: Summary

7.1 General Discussion

The overall aim of the presented thesis was to extensively characterise the rs10305492 human GLP-1R, a low frequency variant (MAF= 1.4%) that is most common amongst Danish Europeans and other non-Finish Europeans (43, 518, 522), in four different models: *in silico*, *in vitro*, *ex vivo* and *in vivo*.

This study was executed after previous observations by GWAS revealed that this variant confers beneficial effects on glucose homeostasis and fasting glucose levels, protecting carriers from getting T2D (43, 518, 522). Moreover, accompanying bioinformatic structural studies showed that the variant alters conformational arrangements in response to endogenous agonists like oxyntomodulin and inter-receptor residue-residue interactions as well as important central polar water networks that are important for G protein engagement (518). Given the favourable impact of this variant on GLP-1R and β -cell function, I speculated that the mutation might result in alterations in conformational rearrangements, residue-residue interactions and receptor dynamics, and that these structural changes in conjunction with trafficking changes may in turn result in altered receptor G protein or β -arrestin-2 recruitment, and ultimately favourable insulintropic and glucose control effects. Investigating this variant in depth could lead to improved understanding of GLP-1R function in relation to structural changes, aiding in the development of enhanced therapeutics. On the other hand, collating pharmacogenomics data on this and other GLP-1R variants contributes to our understanding of interindividual variability in incretin responses. While not currently applicable to guide routine prescribing of incretin therapies, as these are typically made based on clinical response

rather than genotype, such insights may eventually inform subgroup analyses in clinical trials, and possibly support precision medicine approaches in more complex or treatment-resistant cases. Additionally, this study establishes a consensus workflow for the investigation of other GLP-1R variants, paving the way for the design of precision medicine frameworks. Future efforts may also benefit from integrating pharmacogenomic data with pharmacokinetics, adherence profiles, and treatment outcomes to improve therapeutic matching and stratification. Over the last few decades, the association of genetic variation in druggable-GPCR target genes on altered therapeutic efficacy, drug safety and clinical phenotypes, has come to light (459, 506). Indeed, much effort has been made to elucidate and highlight new genotype-phenotype associations in incretin receptors such as the GLP-1R, GIPR, GCGR and many other GPCRs, specifically in the endocrinology, diabetes and obesity fields (459, 506). The novel introduction of databases such as the gene-based association summary statistics (Genebase) (566) and the genome aggregation database (gnomAD) (528) encompassing over 350,000 individual single variant data and exome sequences from the UK biobank (Genebase) as well as from individuals of diverse ancestry (gnomAD) along with their correlated phenotype annotations has greatly aided in identification of important candidate single nucleotide polymorphisms for further extensive studies. GPCRdb comprises another GPCR-specific dataset listing all known natural single nucleotide polymorphisms of GPCR genes and known associated or predicted phenotypes (567).

As previously mentioned, the effects of the A316T mutation on GLP-1R function and pharmacological responses, as well as β -cell function are still unclear due to inconsistencies in available results from different studies. Whilst several studies suggest a GoF effect on GLP-1R signalling and function despite the reduced membrane localisation in HEK, CHO and INS-

1 cells overexpressing the mutant receptor, other contradictory findings in MIN6 murine β -cells reveals that the mutation enhances β -cell apoptosis and reduces GSIS (293, 635, 636).

My study reveals major findings which support the hypothesised GoF effect the human rs10305492 (A316T) GLP-1R variant, translating to favourable glucose metabolism and insulinotropic outcomes in the pancreas, but reveal reduced responses to pharmacological GLP-1RAs.

In silico studies

Firstly, MD simulations reveal that the mutant receptor adopts intermediate active-state conformational rearrangements in a ligand-free form. These include active-state specific conformational rearrangements such as increased opening of the ECD, reduced distance between TM6-TM7 and increased outward swinging of the TM6 kink.

Interestingly, modest alterations in receptor dynamics and residue-residue interactions were found in the GLP-1R (A316T) structure when bound to GLP-1R agonists, GLP1, exendin-4 and its analogue exendin-F1 and exendin-D3, providing insights into the pharmacological signalling and trafficking profile of the mutant receptor. For instance, hydrogen bond networks and increased interaction of the peptide with ECL3 in the presence of exendin-F1 could account for the increased $G\alpha_s$ coupling bias of the GLP-1R (A316T) when bound to this agonist. Other subtle changes include increased flexibility and mobility in the central TM5 area in the presence of exendin-D3, which hints at faster peptide cycling (disengagement and re-engagement) compared to the wild-type receptor.

The cryo-EM structure of the GLP-1R A316T variant bound to GLP-1 and $G\alpha_s$ provides further structural insights, showing a similar static structure to that of the WT GLP-1R but

with subtle changes in interactions between transmembrane helices (TM) 3, 5 and 6. Previous studies based on the X-ray crystal structure of the exendin-4 -bound GLP-1R suggested that the A316T mutation disrupts a hydrogen bond between N320^{5.50} and E364^{6.53} via the formation of a direct interaction between T316 and E364^{6.53}. Yet, the cryo-EM structure of the GLP-1-bound A316T GLP-1R obtained in this study does not exhibit these structural changes, instead suggesting, as supported by MD simulations, a potential disruption to the central polar network residues via subtle alterations in an important interhelical water network bridging TM5 and 6. Additionally, consistent with the mechanisms suggested by the MD simulations, the cryo-EM structure offers support for the formation of a novel hydrogen bond between T316^{5.46} and Y242^{3.45}, replacing P312^{5.42}, selective for an A316T specific rotameric state.

Interestingly, 3DVA of the GLP-1R A316T structure also suggests greater mobility in ECL3, compared to the WT receptor bound to the same agonist, and likely eliciting a similar effect as changes in the central TM5 area; faster peptide cycling, and so more frequent signalling events than with the WT GLP-1R.

Despite these observations, the modest resolution of 3.3 Å for the A316T structure limits the precise placement of structural water molecules that are crucial to understand the proposed mechanism of constitutive activity. MD simulations, which are able to effectively model water molecules peptide structures, identified a network facilitating communication between TM5 and 6 in the A316T GLP-1R, which may contribute to the variant receptor distinct pharmacology. Additionally, these simulations also suggested an allosteric mechanism involving the Y242^{3.45}-T316^{5.46} hydrogen bond influencing TM6 mobility in the apo-state and propagating changes to the intracellular side.

Currently, to my knowledge, there are no structural data available for other constitutively active GLP-1R mutants, making it difficult to fully interpret the impact of the altered water networks and ECL dynamics. For a more comprehensive understanding of the structural alterations driving A316T constitutive activity, higher resolution structures are needed. Also, given that the data suggests that the A316T mutations enhances intrinsic receptor activity, independent of ligand binding, apo-state structures are needed to further explore these differences.

The combined cryo-EM and MD results propose a coordinated mechanism of basal A316T partial activation, driven by the larger and more polar side chain of the T316 residue. This model suggests an allosteric interaction between TM3 and TM5 at position 316 that is transmitted to the intracellular side. The stabilizing role of structural water molecules around the TM6 kink, together with the convergence of TM3 and TM5, appears to facilitate TM6 outward movement, which is essential for $G\alpha_s$ recruitment and activation. At physiological thermal conditions, this mechanism likely shifts the equilibrium of the A316T variant towards an active state, promoting constitutive receptor activation and enhanced $G\alpha_s$ coupling. Ultimately, this suggests that such *in silico* structural studies could be utilised effectively to predict variant-associated changes in ligand specific signalling and trafficking profiles.

In vitro studies

Next, I investigated variant-associated *in vitro* changes in GLP-1R action such as trafficking, signalling and insulin secretion capacity in rodent and human cell lines expressing the SNAP-tagged human GLP-1R.

In both cell lines studied, I detected a significant reduction in plasma membrane localisation of the mutant receptor, consistent with previous *in vitro* observations. In fact, I have also

established in this study that this can be explained by increased basal trafficking and turnover activity, as evidenced by increased endocytosis and lysosomal targeting of the A316T GLP-1R in the absence of an agonist. This trafficking profile is also in agreement with previous hypotheses posed with regards to this variant, as well as with the higher degree of constitutive activity conferred by the A316T mutation (518, 522, 601). Further data corroborating this effect, includes increased levels of constitutive recruitment to lipid rafts and reduced levels of basal receptor plasma membrane diffusion rates for the A316T GLP-1R, both characteristics of agonist-induced WT GLP-1R activation, and thus indicate a higher degree of constitutive activity of the A316T GLP-1R.

As the variant is associated with structural changes in residue networks that may be involved with G-protein and β -arrestin 2 binding and recruitment, I used the previously established NanoBiT complementation assays to assess basal and agonist-induced signalling activity of the variant receptor (362). This revealed that the A316T receptor inherently enhances mini- G_s recruitment, the main activation-associated signalling pathway for the GLP-1R, yet there was no change in basal β -arrestin 2 recruitment, despite its increased endocytosis and lysosomal targeting profile. These results corroborate our previous findings that, despite its apparent correlation with endocytosis, β -arrestin 2 is not required for GLP-1R internalisation (362). On the other hand, alterations in agonist-induced responses to either factor were modest, with a tendency for increased mini- G_s recruitment in response to exendin-F1, and a mild decrease in β -arrestin 2 recruitment relative to the wild-type receptor.

Moreover, bias calculations between these two factors revealed that A316T GLP-1R exhibits exaggerated $G\alpha_s$ bias *versus* β -arrestin 2 in response to exendin-F1, the exendin-4 analogue that itself is already inherently $G\alpha_s$ -biased. This matches with the enhanced A316T-associated

cAMP responses to exendin-F1 in islets. Parallel to the observations in isolated islets, inhibition of lysosomal targeting in cells restored membrane expression of the A316T receptor, further consolidating the increased lysosomal trafficking theory.

Protein interactome studies of the A316T GLP-1R also corroborated the increased inherent active profile of this receptor, with a marked increase in interactions compared to the wild-type GLP-1R in vehicle conditions, resembling those of the active WT GLP-1R (540). A prominent exemplar is the mutant GLP-1R engagement with VAPB, recently shown to engage the GLP-1R at ER MCSs mediating mitochondrial remodelling in β -cells (540). Interestingly, here I observe that contrary to the WT GLP-1R, the A316T GLP-1R exhibits reduced engagement to VAPB in response to agonist stimulation but enhanced basal engagement with this interactor. This may suggest that the mutant receptor might be less able to interact with mitochondrial-ER sites to mediate remodelling in response to GLP-1RAs, as interactions with VAPB are less potent, or that the receptor is already engaged in this nanodomain in the absence of agonist stimulation.

As previously shown by our group, the WT GLP-1R interactomic profile provides important insights into the main pathways engaged by the receptor to execute its crucial functions in β -cells (566). Thus, individuals with specific mutations in the receptor, such as A316T, are likely to exhibit differential interactomic profiles, as well as distinct-agonist dependent profiles, adding complexity to the pathways engaged by the receptor.

In terms of other downstream functional assessments of the A316T variant, I assessed insulin secretion in a human β -cell line revealing a similar pharmacological profile as seen in the various *ex vivo* human and mouse islet models, with significantly enhanced levels of GSIS triggered by the A316T variant independent of agonists. In agreement with the *in vivo* results,

insulin secretion responses to GLP-1RAs were impaired, with prolonged exendin-4 fold-responses particularly affected, a detrimental effect that matches increased plasma glucose levels assessed at 6 hours post-exendin-4 administration.

Similarly, agonist-induced cAMP and intracellular Ca^{2+} responses, specifically to exendin-4 were impaired, in line with the previous insulin secretion findings *in vitro* and *ex vivo*, as well as recent studies showing limited cAMP responses to exendin-4 and semaglutide in cells (636). Only in the presence of the biased agonist exendin-F1 was the variant receptor functioning at an efficiency resembling or better than its apostate form. I hypothesise that this effect might be linked to the favourable trafficking profile of this biased GLP-1R, enabling it to counteract the accelerated desensitisation propensity of the A316T GLP-1R. Alternatively, this could also be explained by the shared ECL3- β -arrestin 2 associated conformational changes occurring in the A316T and wild-type bound exendin-F1, which may result in additive or sustained effects after the addition of this agonist (293, 303, 304, 384, 562). On the other hand, the A316T variant likely exacerbates the fast internalising, slow recycling trafficking profile of the exendin-4 agonist, this along with enhanced exendin-4 induced ubiquitination of A316T versus the WT GLP-1R, a PTM traditionally associated with receptor lysosomal targeting, degradation and desensitisation (370, 371), contributing to impairments in glucoregulation, particularly at prolonged time points.

In vivo studies

I have designed a novel knock-in mouse model bearing the A316T mutation in the human *Glp1r* locus with glycaemic traits resembling those observed in GWAS studies (43, 518, 522). In this knock-in *in vivo* model I observed significantly positive effects of the variant on oral glucose tolerance under both the chow and HFHSD conditions, suggesting enhanced

sensitivity to the endogenous GLP-1, as well as significantly lower fasting glucose levels in the chow fed state in agreement with the GWAS studies in humans (43, 522). Interestingly, the variant also exhibited slight anorectic effects in the diet induced obesity mice, where the rate of weight gain was reduced over a 12-14-week period despite similar initial starting weights, indicating a protective role against obesity. Notably, this anorectic effect of the A316T variant was measurable despite the reduced human GLP-1R expression levels in my humanised mouse model compared to non-humanised mice, which given the limited *Glp1r* expression in the brain may mask any observable effects relating to appetite regulation, meaning I may even be underestimating these anorectic effects.

Despite the positive effects of the variant on glucose homeostasis and endogenous GLP-1 responses, I found that it reduces the efficacy of responses to pharmacological GLP-1RAs like exendin-4, relative to its effect under vehicle conditions. Specifically, sustained exendin-4 responses were particularly impaired, to the point of eliciting a worse response compared to vehicle in chow fed *hGlp1r^{A316T/A316T}* mice; while exendin-F1, the $G\alpha_s$ biased analogue of exendin-4, retained its sustained responses relative to vehicle conditions despite an initial loss of effect acutely in chow-fed *hGlp1r^{A316T/A316T}* mice compared to wild-type littermate controls. This identified *in vivo* phenotype is concomitant with increases in plasma insulin and islet insulin secretion levels from A316T lean and obese mice under vehicle conditions in both and obese *hGlp1r^{A316T/A316T}* mice and are likely a result of increased constitutive GLP-1R activity mimicking agonist-induced effects in the pancreas. In parallel to glucose tolerance findings, plasma insulin levels also indicate changes in insulin secretion both basally and in response to GLP-1RA administration compared to the WT control mice.

While the A316T variant maintains its positive effects on lowering glucose levels in HFHSD-fed mice, loss of agonist-induced responses is exacerbated relative to vehicle conditions regardless of the agonist administered.

I have also established the clinical relevance of my observations, as the two currently leading pharmacological GLP-1RAs agonists, semaglutide and tirzepatide, elicit similarly reduced responses in *hGlp1r*^{A316T/A316T} HFHSD-fed mice, an effect that is also present in heterozygous *hGlp1r*^{A316T/+}, the most likely form of variant due to its low frequency in human populations.

Together, these findings imply that the A316T variant's glucoregulatory enhancing phenotype is present even under metabolic stress, but that pharmacological responses vary depending on the metabolic state and agonist used. Overall, more experiments are required to determine whether these A316T specific pharmacological effects can be rescued with chronic administration of the agonists or with the administration of different agonist doses. Also, the effect of the constitutively active A316T GLP-1R on metabolic glucoregulation via gut hormone secretion should be further investigated as an explanation for mechanisms underlying improved oral glucose tolerance. Moreover, although the desensitising effect of exogenous GLP-1RAs was studied via the IPGTTs, OGTTs were not conducted over longer time-points, to establish if there is also a desensitisation effect conferred by the endogenous GLP-1 hormone on the A316T GLP-1R.

Ex vivo studies

In addition to the *in vivo* study, I have also performed a comprehensive *ex vivo* analysis of the variant effects on islet gene expression, islet morphology, and α - versus β -cell mass. In parallel to HFHSD-diet specific alterations to A316T GLP-1R pharmacological responses, the islet cellular architecture was also altered with significantly increased islet diameter and α -cell

mass, with an interesting increase in core localisation of these α -cells only under metabolic stress conditions. Furthermore, I observed the presence of increased bi-hormonal cells expressing both insulin and glucagon in the HFHSD-fed A316T mice, the presence of which has been associated with human islet responses to GLP1RA treatment (219, 637). Similarly, HFD feeding and metabolic stress has been previously shown to induce β -cell like changes in a subset of α -cells, and increased α -cell derived GLP-1 secretion (220, 229). A possible explanation for this is that the A316T variant might alter islet morphology and cyto-architecture under metabolic stress conditions as a compensation mechanism, resulting in altered islet hormone expression including GLP-1 or glucagon and thus intra-islet paracrine signalling. Yet, despite changes in islet morphology, I observed only mild increases in β -cell enriched gene expression such as *Mafa*, and a tendency for increased expression of *Ins1* and *Kcnj11*, in *hGlp1r*^{A316T/A316T} islets under both chow and HFHSD-fed conditions.

To further my knowledge of the mechanisms underlying the changes in *in vivo* glucoregulation observed in the presence of the A316T variant, I assessed GSIS and cAMP signalling patterns in intact knock-in *hGlp1r*^{A316T/A316T} islets and observed an insulin secretion profile in agreement with my *in vivo* results. Insulin secretion from HFHSD-fed *hGlp1r*^{A316T/A316T} mouse islets in response to glucose alone was significantly enhanced relative to the wild-type islets, yet these islets exhibited a defective response to GLP-1RA exendin-4 after both acute and sustained simulations relative to the response to glucose alone.

In addition, I also measured acute cAMP signalling responses to GLP-1RAs, again showing that reductions in acute exendin-4-stimulated insulin secretion in islets expressing the A316T mutation correlate with reductions in cAMP responses for some agonists. On the other hand, the $G\alpha_s$ -biased exendin-4 analogue, exendin-F1, resulted in a lower degree of insulin secretion

dampening relative to 11 mM glucose alone, and exhibited a modestly increased cAMP response. I also assessed GLP-1R trafficking and cell surface expression patterns in intact islets, and in agreement with previous studies *in vitro*, there was a significant reduction in cell surface expression of the A316T GLP-1R compared to the wild-type.

In addition, using an *in vivo* technique involving injection of Luxendin645 into living mice, I could detect a loss of cell surface expression of the endogenous GLP-1R in the pancreata of *hGlp1r*^{A316T/A316T} compared to wild-type controls in agreement with isolated islet data. Reduced cell surface expression of the variant receptor was found to be due to increased basal lysosomal targeting and endocytosis, suggesting that the inactive receptor has a higher turnover rate and thus increased basal trafficking activity.

Most of my observations so far were done in mouse islets, but I have also tested insulin secretion and membrane localisation of the variant receptor and lysosomal trafficking in human islet models via transduction of adenoviruses expressing the SNAP-tagged wild-type *versus* variant receptors. Parallel to my findings in the knock-in mouse islets and β -cell lines, I found a significant reduction in plasma membrane localisation of the A316T GLP-1R in both human islets and human β -cells models relative to the wild-type receptor. Furthermore, insulin secretion results observed also resembled those seen in the mouse islets, with a significant enhancement in the absence of ligands and a defective response to exendin-4 stimulation acutely relative to glucose responses alone. While the human islets retained wild-type GLP-1R expression, my findings in agreement with mouse islet results show that the GoF phenotype of the A316T receptor is not limited to one model, and the effects are strong enough to elicit

an enhanced insulinotropic phenotype even in the presence of the wild-type receptor in heterozygous models.

7.2 Limitations and Future Perspectives

A limitation of my study is that data *in vivo* responses were collected from a mixed sex cohort and would have been more informative if collected from separate male and female mouse cohorts. Indeed, the rs10305492 variant is more frequent in the female population. Thus, future studies should include more male and female genetically engineered animals to test if there are any sex-specific variant associated effects in comparison to wild-type animals. Furthermore, an important limitation of my genetically engineered mouse model is the parental mouse line used in its generation, the *hGlp1r*^{+/+} line.

During my *in vivo* studies, I discovered that mice belonging to this line required increased doses of GLP-1RAs to elicit a similar response to the same agonist in normal non-transgenic mice due to the fact that these mice express significantly less GLP-1R on their cell surface. This is believed to be a by-product of the generational design of these mice, by which the hGLP-1R cDNA comprising only exonic regions, is inserted downstream of exon 2 of the mGLP-1R and no intronic regions which are important for regulating the expression of the GLP-1R are included. Although, in this case this did not pose a big issue as my aim was to compare *hGlp1r*^{A316T/A316T} mice with their wild type *hGlp1r*^{+/+} littermates, which are both derived from the same parental line and so have the same inherent limitation, I predict that the effects of the mutation would be more pronounced in a refined *hGlp1r*^{+/+} mouse which includes

all intronic regions. Hence, future studies should include generation and characterisation of these mice, then a repeat of my *in vivo* studies in these animals to test this hypothesis.

Finally, as I observed a change in weight gain on the HFHS diet, additional studies examining the anorectic effect of the mutation, including food intake and appetite regulation in brain regions are warranted to corroborate phenotypes specific for metabolic stress conditions induced by the HFHSD. Moreover, GLP-1R also plays an important role in gastric emptying which is another important regulator of glucose metabolism. Thus, these factors should be studied to understand the full effects of the variant on the regulation of glucose control and how this contributes to the T2D protective phenotype association with this mutation.

GLP-1R is localised in organs other than the pancreas, and I am using a whole-body knock-in mouse model, it is imperative to assess the effects of the rs10305492 variant in other organs such as the brain and the heart. GLP-1R localised in brain regions, such as the brain stem and the hypothalamus, plays an important role in regulating energy homeostasis, including appetite, food intake and body weight. If the A316T variant inherently increases the receptor function in the brain, it could influence the efficacy and safety profile of other GLP-1RAs when administered at normal doses which could lead to adverse effects such as nausea and reduced drug efficacy. Hence, investigating the effects of this mutation on GLP-1R function in tissue isolated from the knock-in *hGlp1r*^{A316T/A316T} mice including hypothalamic neurons could help us understand its consequences on processes such as neuronal activity, neurotransmitter release, food intake and energy expenditure. Importantly, GWAS studies have shown that the rs10305492 mutation is also associated with protection against cardiovascular disease. Moreover, the endogenous GLP-1RA, GLP-1, is involved in the regulation of heart rate and blood pressure. Similarly, other GLP-1RAs have also been found

to be involved in reducing ischemic injury by reducing cardiac cell apoptosis, enhancing cell survival pathways and improving cardiac function.

Therefore, it is imperative to study the precise mechanisms by which this mutation promotes beneficial cardioprotective effects, to aid in the development of advanced therapeutics for heart disease. As for the brain, this can be achieved by assessing GLP-1R function in tissue or cells isolated from knock-in *hGlp1r*^{A316T/A316T} mice such as cardiomyocytes or parasympathetic neurons innervating the heart.

In parallel to this, although my study provides an almost-exclusive β -cell-centric assessment of the impact of the rs10305492 GLP-1R variant, it neglects to do the same for other islet cell types such as α - and δ - cells, the function of which has been increasingly gaining attention. The role of this GLP-1R variant in α -cells is of particular interest to me, as I observed a striking α -cell mass increase in *hGlp1r*^{A316T/A316T} islets under metabolic stress conditions. This α -cell hyperplasia may reflect the possibility of hepatic glucagon resistance driven by over-suppression of glucagon secretion due to constitutively active A316T GLP-1R. This could trigger a compensatory liver-islet feedback loop, wherein increasing circulating amino acids from the liver and stimulating α -cell expansion to compensate for reduced glucagon signalling. Such a mechanism may serve to prevent hypoglycaemia by re-establishing adequate glucagonotropic tone. Investigating liver-specific signalling, amino acid metabolism, and glucagon secretion in future studies could further substantiate this liver- α -cell hypothesis in the context of the A316T variant.

Not only the abundance, but also the localisation of these α -cells was altered with an observed transition from the periphery to the core of the islet, resembling human islet cyto-architectures.

Evidence from previous studies has shown that chronic hyperglycaemia provokes changes in islet cyto-architecture, including α -cell hyperplasia and β -cell de-differentiation into glucagon-positive producing cells (229). Conversely, treatment with GLP-1RAs is associated with the opposite effect, which makes our results perplexing as the variant increases GLP-1R function and mimics the action of these agonists (229). This phenomenon could be an attempt at a compensatory mechanism involving alterations of paracrine cell-cell interactions as well as alterations in non β -cell hormone secretion like glucagon and GLP-1 (229).

From a pharmacological perspective, the A316T associated α -cell hyperplasia observed under metabolic stress conditions likely contributes to elevated circulating glucagon levels as well as impaired responses to the dual GLP-1R/GIPR agonist tirzepatide *in vivo*, which were notably worse compared to the biased GLP-1 agonist exendin-F1. Tirzepatide, which incorporates a GIPR component, might exacerbate the hyperglycaemia due to the presence of an increased number of hyperactive α -cells, resulting in excessive glucagon secretion and GIPR mediated effect. This in turn may overwhelm the glucose-lowering effects of the biased GLP-1R component of tirzepatide, particularly in the context of insufficient compensatory insulin secretion. Conversely, exendin-F1, which specifically targets β -cells, may sidestep the detrimental effects of α -cell hyperplasia. Henceforth, it would be valuable for future experiments to investigate whether treatment of HFHSD-fed *hGlp1r*^{A316T/A316T} mice with GIP agonists such as GIP-dAla2, which stimulate both β -cell insulin and α -cell glucagon secretion will exacerbate hyperglycaemia, especially in the context of α -cell hyperplasia. On the other hand, excess glucagon may lead to overaction of the GCGR, driving diverse metabolic effects including lipolysis, ketogenesis, and hepatic amino acid uptake, as well as anorectic effects such as reduced food uptake and increased energy expenditure (638). While some of these

effects may benefit fatty liver disease and energy metabolism, overactivation of GCGR also exacerbates hepatic glucose output and impairs glycaemic control. This emphasizes the need to evaluate the balance between GIPR and GLP-1R signalling in therapeutic strategies, especially in the context of genetic variants.

Additionally, further studies in α -cell models such as the murine α -TC1 cell line replicating experiments done in the rodent INS-1 832/3 cell line and assessment of functional outcomes like glucagon and GLP-1 secretion are warranted to understand the effect of this mutation on α -cell function. Plasma glucagon levels could be assessed, and α -cells could also be isolated from *hGlp1r*^{A316T/A316T} islets for a more physiological approach.

Finally, although human adult islets and EndoC- β H3 cells provided an important opportunity to study this variant in a human context, here are limitations associated with their use including the unpredictable availability of human islets resulting in low power, and the fact that both systems are difficult to genetically manipulate. In terms of my studies in human islets, I have used adenoviral SNAP/FLAG-tagged hGLP-1R^{A316T} or ^{WT} vectors to overexpress the corresponding receptor proteins without genetically altering the endogenous GLP-1R expression itself, thus data from functional studies such as insulin secretion could also be due to interactions with the endogenous GLP-1R. On the other hand, for the EndoC- β H3 I have genetically eliminated GLP-1R expression before transduction with the adenoviral vectors, but unlike human islets, this model does not contain multiple cell types and so cannot be used to assess the effects of the variant on different islet endocrine cell types.

An alternative model combining positive features of both primary adult human islets and the EndoC- β H3 lines might be provided by stem-cell derived islets (SC-islets). They can be

generated from human pluripotent stem cells (hPSCs) either utilising human induced pluripotent stem cells (hiPSCs) or human embryonic stem cells (hESCs) (639). SC-islet generation from hPSCs is a valuable system to study GLP-1R variants, as hPSCs can self-renew and proliferate indefinitely meaning genetic manipulation is easier because single cell colonies are easier to amplify, and they can differentiate into specialised cell types of the three germ layers (639). Although early differentiation protocols yielded SC-islets that were inferior compared to differentiated islets, new protocols closely mimic native human islets in function and transcriptomic profile (639). Furthermore, fluorescent reporters under the control of different islet cell-specific promoters may be used to study the effect of the variant on the development and maturation of different islet cell types. Overall, use of SC-derived islets provides a more comprehensive, and flexible model for studying the effects of the A316T variant, leading to better understanding and potentially improved treatments for T2D including perhaps innovative gene therapies whereby these genetically modified islets are transplanted into diabetic subjects.

7.3 Conclusions

In conclusion, my thesis has identified the rs10305492 human GLP-1R missense mutation as a positive regulator of β -cell function and strongly suggests that it promotes protection against T2D via adopting an intermediate-state ligand free state resulting in a GoF GLP-1R phenotype with exaggerated trafficking, signalling, interactomic and physiological responses independent of ligand.

I present here a comprehensive assessment of the effects of the A316T missense mutation in a structural context using cryo-EM complexes, *in silico* using MD computational studies, in human and rodent pancreatic β -cells, *ex vivo* human and murine islets and *in vivo* genetically engineered mice, including both agonist-independent and dependent responses. I have demonstrated reduced *in vivo* agonist-dependent glucoregulation effects that manifest in islet insulin secretion also. These effects correlate with cAMP responses *in vitro*. My study analyses in detail the agonist-dependent and independent trafficking and coupling responses of this variant in rodent β -cells confirming a markedly GoF phenotype for the A316T missense mutation, with reduced cell surface expression, enhanced G protein coupling and significantly enhanced endocytosis and trafficking. These data have important implications for the design of future GLP-1RAs, whereby structural residue-residue interactions could be emulated to achieve allosteric effects similar to that of the variant, and individuals could be prescribed GLP-1RAs based on their GLP-1R variant profile. In addition to this, my workflow can be adapted to other GLP-1R variants aiding in the establishment of an annotated genotype-phenotype datasets, that can be utilised to advance precision medicine approaches.

7.4 Conclusion diagram

Table 4.1 Overview of effects of the GLP-1R A316T in inactive and active states compared with GLP-1R WT. (increased $\uparrow$, decreased $\downarrow$, $\approx$ unchanged, two arrows=significance)

Assay	GLP-1R A316T versus GLP-1R WT				
	(Vehicle) Inactive	GLP-1	Exendin-4	Exendin-F1	Exendin-D3
Surface Expression	$\downarrow\downarrow$	N/A	N/A	N/A	N/A
Lipid raft Recruitment	$\uparrow\uparrow$	$\approx$	$\downarrow\downarrow$	N/A	N/A
Ubiquitination	$\approx$	N/A	$\uparrow$	N/A	N/A

Internalisation	↑↑	↑	≈	↓	↑
Recycling	N/A	↓	≈	↓	≈
Degradation	↑↑	≈	≈	↓	↑
Clustering	≈	≈	N/A	N/A	N/A
ECD opening	≈	↑↑	N/A	N/A	N/A
Mini-Gs recruitment	↑↑	≈	≈	↑	≈
β – arrestin 2 recruitment	≈	≈	≈	↓	≈
Mini-Gs Bias	N/A	≈	≈	↑	↓
Activation at endosomes	≈	↑	≈	≈	↑
Activation at plasma membrane	≈	≈	≈	≈	↓
Ca²⁺ mobilization	≈ (islets)	≈	≈	↑ (G6) ≈ (G11)	N/A
cAMP production	↑ (islets)	↑ (cells)	↓ (cells and islets)	↑ (islets) ≈ (cells)	≈
Insulin secretion	↑↑ (0hr) ↑↑ (6 hr)	N/A	↓↓ (0 hr) ↓ (6 hr)	↓↓ (0 hr) ↓ (6 hr)	N/A
Glucose tolerance	↑↑ (0 hr) ↑↑ (6 hr)	↑↑ (oral)	↓↓ (0 hr) ↓↓ (6 hr)	↓↓ (0 hr) ≈ (6 hr)	N/A
Plasma Insulin	↑ (0 hr) ↑↑ (6 hr)	N/A	↓ (0 hr) ↓↓ (6 hr)	N/A	N/A

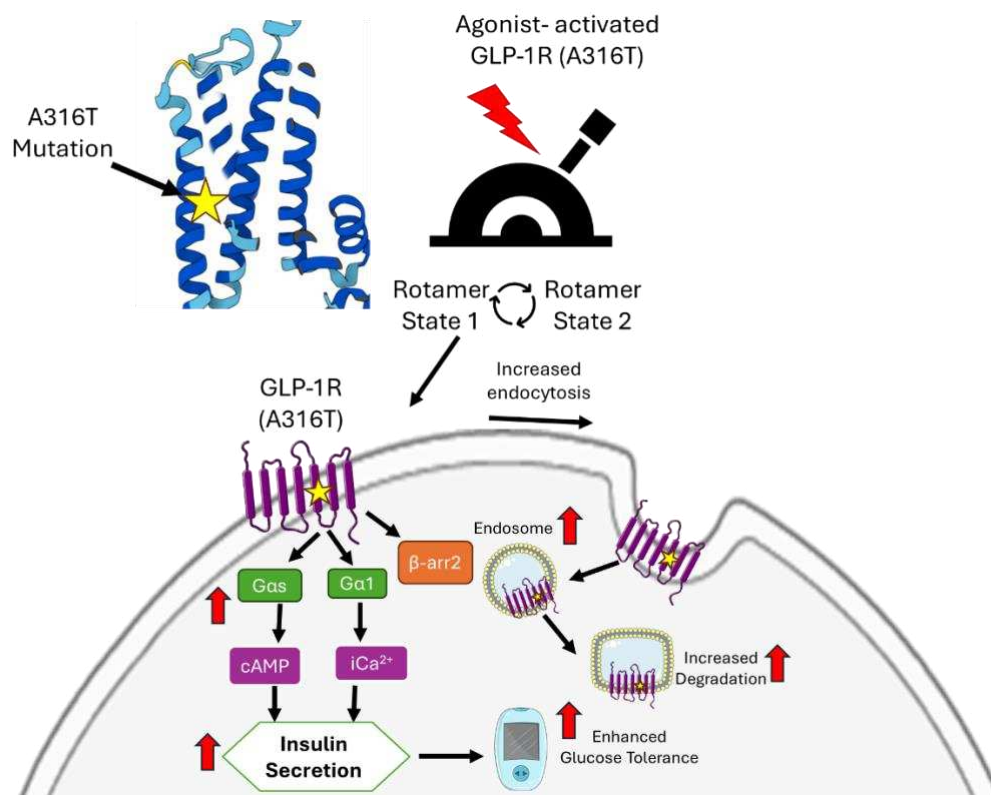
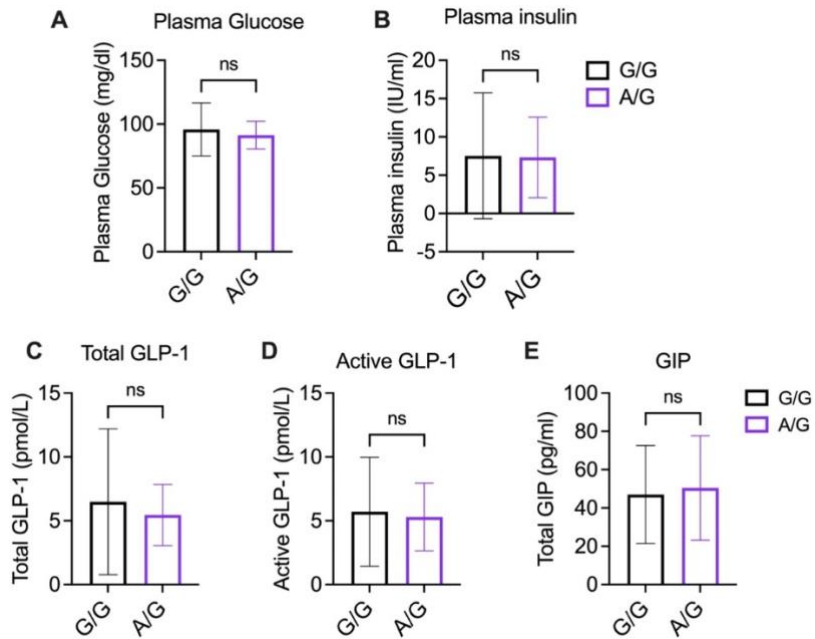


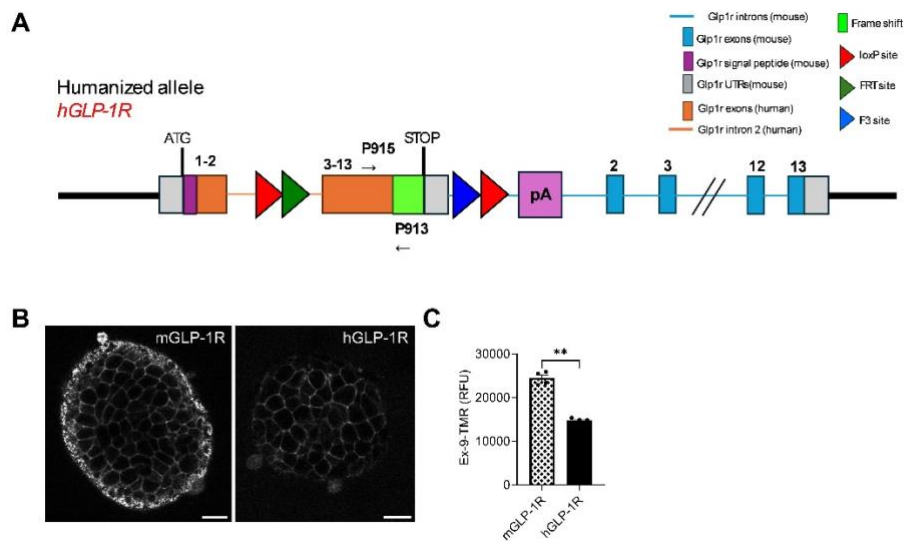
Figure 7.1 Schematic diagram of effects of the rotameric GLP-1R A316T on GLP-1R function. Arrows indicate increased pathway engagement.

Appendices

Appendix 1: Supplementary Figures and Materials



Supplementary Figure 1 Comparison of glycaemic traits and plasma incretin hormone levels between human individuals with G/G and A/G rs10305492 genotype. Data was obtained from(529).



Supplementary Figure 2 Design and surface expression characterisation of the hGlp1r^{+/+} mouse model. A) Schematic representation of the gene that is expressed in the hGlp1r^{+/+} mice. B) Representative fluorescence images from 1mM Ex-9TMR labelled mouse islets from mGLP-1R and hGlp1r^{+/+} mice and corresponding quantification (C). Scale bars represent 50 μ m.

Appendix 2: List of Publications

- **October 2024-** El Eid L., Deane-Alder K., Rujan R.M. *et al.* 'In vivo functional profiling and structural characterisation of the human *Glp1r*^{A316T} variant', [Preprint-bioRxiv].
- **July 2024-** Manchanda, Y. El Eid L., Oqua A. *et al.* 'Engineered mini-G proteins block the internalization of cognate gpcrs and disrupt downstream intracellular signalling', published in *Science Signalling*
- **June 2024-** Oqua, A.I. *et al.* 'Molecular mapping and functional validation of GLP-1R cholesterol binding sites in pancreatic beta cells', published in *eLife*.
- **April 2024-** Austin, G. *et al.* 'An inter-organelle contact between endosomal GLP-1R, er vap-B, and the mitochondrial AKAP SPHKAP triggers PKA-dependent MIC19 phosphorylation and β -cell mitochondrial remodelling', [Preprint-bioRxiv].
- **October 2023-** Hinds, C.E. *et al.* 'Abolishing β -arrestin recruitment is necessary for the full metabolic benefits of G protein-biased glucagon-like peptide-1 receptor agonists', published in *Diabetes, Obesity and Metabolism*, 26(1), pp. 65–77.
- **May 2023-** Bitsi S., El Eid L., Manchanda Y., I. Oqua A., Mohamed N., Hansen B., Suba K., Rutter A. G, Salem V., Jones B., and Tomas A, 'Divergent acute versus prolonged pharmacological GLP-1R responses in adult β cell-specific β -arrestin 2 knockout mice,' published in *Science Advances*
- **April 2023-** Xiao J., El Eid L., Buenventura T., Boutry R., Bonnefond A., Jones B., Rutter G., Froguel P., and Tomas A., 'Control of human pancreatic beta cell kinome by GLP-1R biased agonism', published in *Diabetes, Obesity and Metabolism*
- **October 2022-** EL Eid L., Reynolds A. C., Tomas A., and Jones B., 'Biased agonism and polymorphic variation at the GLP-1R: Implications for the development of personalized therapeutics,' published in *Pharmacological Research*

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