FUNCTIONAL CHARACTERIZATION OF DIABETES- AND GLYCEMIC TRAIT-ASSOCIATED SNPs IN GLP-1R

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ABSTRACT

There is increasing evidence that human genetics may be able to improve the development of antidiabetic therapies by helping to select the best drug targets by predicting potential drug efficacy and toxicity. Recent targeted exome sequencing has identified potentially functional exonic variants in six genes encoding drug targets for the treatment of obesity and type 2 diabetes (T2D). We focused our work on the low-frequency missense variant (Ala316Thr; rs10305492) in the gene encoding glucagon-like peptide-1 receptor (GLP-1R). GLP-1R is the target of GLP-1 agonists, long-acting mimetics of the incretin hormone GLP-1, which increase insulin secretion by pancreatic β-cells following oral consumption of glucose but not after administered intravenously glucose. Unlike most other variants identified for T2D, rs10305492 is associated with lower fasting glucose levels and lower T2D risk as well as with reduced risk of developing coronary heart disease. We therefore investigated the molecular pathway by which this variant could influence metabolic traits.

To better understand how rs10305492 changes the mechanism of action of GLP-1R in pancreatic β-cells, the detailed study was performed. We first examined the cell membrane expression and activity of human wild type and mutant GLP-1R using FACS, live cell imaging and BRET assays. Interestingly, despite having low cell surface membrane expression, the mutant receptor GLP-1R (A316T) showed the same cAMP accumulation and internalization activity as the wild type receptor. However, the mutant receptor resulted in decreased Ca^{2+} mobilization and severely blunted β-arrestin2 recruitment. Taken together, our data has shown that the genetic GLP-1R variant which is associated with low T2D risk induces signalling bias by stabilizing a receptor conformation that couples differentially to intracellular signalling pathways compared to the wild-type receptor.

Additional studies should be performed to confirm our hypothesis however our results suggest that the rs10305492 variant and possibly other genetic variants associated with metabolic traits can be used to develop assays to validate therapeutic targets at an early stage in the drug development process. Our findings also highlight the potential utility of biased agonists as novel GLP-1R therapeutics.

Key words: G-protein coupled receptors (GPCR), glucagon-like peptide-1 (GLP), glucagon-like peptide-1 receptor (GLP-1R), gastric inhibitory peptide receptor (GIPR), Type 2

Diabetes Mellitus, single nucleotide polymorphism (SNP), bioluminescence resonance transfer (BRET), GPCR signalling bias, positive allosteric modulators (PAM).

ABSTRACT (French)

L'avancement des connaissances de génétique humaine contribue au développement de thérapies antidiabétiques visant à sélectionner les nouvelles cibles, prédire l'efficacité et la toxicité du médicament potentiel.

Récemment, le séquençage d'exomes a identifié plusieurs variantes dans 6 gènes impliqués dans le développement de l'obésité et le diabète de type 2 (DT2) et qui pourraient être des cibles thérapeutiques. Parmi ces variantes génétiques, la variante rare (Ala316Thr; rs10305492) a été identifiée dans le gène codant le récepteur du glucagon-like peptide-1 (GLP-1R). GLP-1R est la cible des analogues du GLP-1, des mimétiques d'incrétines à action de longue durée. Les incrétines sont des hormones qui entraînent une production et sécrétion accrue d'insuline par des cellules β du pancréas après la consommation orale de glucose, et non après l'administration de glucose par la voie intraveineuse. Contrairement à la plupart des variantes associées au développement du DT2, rs10305492 est associé à un taux de glucose à jeun faible et réduit le risque de développer le DT2 et la maladie coronarienne. Nous avons donc étudié la voie moléculaire par laquelle cette variante pourrait influencer les traits métaboliques.

Nous avons examiné l'expression de GLP-1R de type sauvage et GLP-1R mutant (A316T) à la surface cellulaire et comparé leurs activités. Nous avons employé un trieur de cellules à fluorescence (fluorescence-activated cell sorter - FACS), de l'imagerie de cellules vivantes et de l'imagerie par bioluminescence (bioluminescence resonance energy transfer - BRET). Nous avons démontré que malgré une faible expression à la surface cellulaire du mutant GLP-1R (A316T) il y avait une accumulation d'AMPc semblable à celui du récepteur de type sauvage. L'activité d'internalisation était aussi comparable à celle du GLP-1R de type sauvage. Cependant, le recrutement de β-arrestine 2 et la mobilisation de $_{i}Ca^{2+}$ pour cette variante étaient sévèrement diminués. Prises ensemble, nos données démontrent que la variante génétique GLP-1R (A316T) qui est associée à un faible risque de DT2 induit une signalisation biaisée, qui privilégie différentiellement des voies de signalisation, en modulant la conformation du récepteur.

Bien que des études supplémentaires doivent être effectuées pour confirmer notre hypothèse, nos résultats suggèrent que la variante rs10305492 ainsi que d'autres variantes génétiques associées à des traits métaboliques et des maladies multiples peuvent être utilisés pour développer des tests validant des cibles thérapeutiques à un stade préliminaire dans le

processus de développement de médicaments. Nos résultats mettent également en évidence l'utilité potentielle des agonistes biaisés de GLP-1R comme de nouvelles thérapies antidiabétiques.

Mots clés : Récepteurs couplés aux protéines G (GPCR), glucagon-like peptide-1 (GLP-1), récepteur du glucagon-like peptide-1 (GLP-1R), récepteur au peptide insulinotrope dépendant de glucose (GIPR), diabète de type 2, polymorphisme d'un seul nucléotide (SNP), transfert d'énergie de résonance de bioluminescence (BRET), signalisation biaisée, modulateurs allostériques positifs des récepteurs (PAM).

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Introduction

In recent years, the prevalence of Type 2 Diabetes Mellitus (T2D) has risen dramatically worldwide. T2D results from impaired insulin secretion by pancreatic β-cells and insulin resistance in peripheral tissues. Current treatments are effective in reversing many of the symptoms of T2D; however, they are not able to stop the progression of β-cell failure or the effects of diabetes on other tissues which include high risk of cardiovascular disease, stroke, retinopathy, nephropathy and neuropathy. Therefore, new therapies for diabetes are being developed that would maintain normal levels of insulin secretion by stopping the progression of β-cell damage and improving β-cell function.

Recently, the incretin hormone GLP-1 and its receptor GLP-1R have been successfully introduced as targets for T2D therapy. GLP-1 stimulates insulin secretion through activation of GLP-1R in response to food intake. GLP-1R-based therapy has several advantages over other existing treatments since it targets two important metabolic regulatory pathways by increasing insulin secretion and decreasing glucagon levels and has the potential to affect β-cell mass. Several GLP-1 analogues have been developed; and two (exenatide and liraglutide) have been already approved to be used in T2D therapy [1]. These therapies have been associated with weight loss [2] and reduced cardiovascular risk factors [3]. However, clinical trials evaluating the cardiovascular safety of GLP-1R agonists (to meet new US FDA requirements [4]) have not yet been completed [5].

GLP-1R belongs to the family B GPCRs and is known to couple to multiple effectors through $G\alpha_s$- and $G\alpha_q$-mediated [6] as well as G protein independent (β-arrestin mediated) pathways [7, 8]; and *in vivo* data support the importance of these signalling pathways for normal homeostasis in both glucose and energy metabolism [9]. The multiple ligands of GLP-1R have been shown to have biased signalling effects that promote different cellular responses [10, 11]. The main GLP-1R signalling pathway is through the $G\alpha_s$ protein and leads to cAMP production [12]; while other GLP-1R interactions lead to Ca^{2+} mobilization and phosphorylation of ERK1/2 [11]. The contribution of these signalling pathways to the beneficial action of GLP-1R and the extent to which one is activated relative to another is therefore important for development of new therapeutics.

The complexity of the ligand-GLP-1R signalling pathway is increased by the presence of several nonsynonymous single nucleotide polymorphisms (SNPs) that occur naturally in GLP-1R and that can alter its signalling activity [12]. Recently, a mutation (rs10305492) has

been described in GLP-1R that affects diabetes susceptibility [13]. Interestingly, rs10305492 (A316T; MAF = 1.4%) reduces rather than increases T2D risk; and normoglycemic patients who carry the risk-reducing allele have lower fasting glucose levels and reduced early insulin secretion. The low-frequency variant protective for T2D has been also found to be protective for coronary heart disease (CHD) [14]. Studies suggest that this minor allele is a gain-of-function mutation that mimics the effect of GLP-1R agonist therapy and may be used for predicting the mechanism of action and possible adverse effects of drug candidates aiming GLP-1R in diabetes therapy.

During the last decade, genetic variants associated with common diseases have been intensively studied for their use in drug design [15]. Naturally occurring mutations affecting the activity of particular protein target in drug discovery can be used to predict the potential drug efficacy and toxicity at the stage of target validation, much earlier than phase III clinical trials, saving considerable time and money [16]. Also, these variants have been shown to provide important information about the treatment or prevention of human disease reducing the prioritisation of potential targets or implicating new targets [16, 17].

Deep understanding of functional consequences of variants is required to evaluate the utility of the mutations in target validation. However, there is not much data about the effect of the GLP-1R variant rs10305492 in cellular models. Using FACS, live cell imaging and different BRET assays, we investigated the signalling profile of mutant GLP-1R (A316T). Our data show that this mutation induces signalling bias by stabilizing a receptor conformation that preferentially interacts with $G\alpha_s$. The concept of signalling bias is similar to ligand-directed signalling or biased agonism whereby different ligands stabilize unique receptor conformations. Understanding the functional outcome of the gain-of function minor GLP-1R allele could lead to the rational design of biased GLP-1R agonists capable of activating a therapeutically important signalling pathway over those leading to other (possibly harmful) physiological effects. This study also demonstrates how genetic target validation approaches can be employed early in the drug development process to evaluate efficacy and safety; which becomes particularly important given the recent reports of increased risk of pancreatitis associated with GLP-1 mimetics and the observed clinical differences between liraglutide, exenatide and the recently-developed long-acting formulation of exenatide [18].

Rationale and Hypothesis of the Thesis

Recent findings showed that a low-frequency missense variant (Ala316Thr; rs10305492) in the gene encoding GLP1R, an important target for treatment of type 2 diabetes mellitus, was associated with lower fasting glucose and lower T2D risk. The fact that this variant mirrors the effect of the clinically used GLP-1R agonists attracted the attention of the researchers because of its potential application in drug design. However, before mutant receptors can be used for target validation, the functional consequences of the genetic variant should be studied and understood. This knowledge will not only provide an insight into the effects that the mutation has on the receptor function but also will help to understand its possible association with the onset of disease or effectiveness of drug therapies.

Understanding of the mechanism of action of the mutant receptor may also serve as starting point for novel strategies to enhance GLP-1R activity and thus its therapeutic potential. Furthermore, characterizing the mutant GLP-1R function by means of BRET may provide valuable insights into understanding signalling of GPCR family B members in general, advancing the field of GPCR research. It is, therefore, important to study the changes in GLP-1R (A316T) downstream pathway engagement, which may help to understand the increasingly diverse actions of GLP-1 and the complex nature of GLP-1R signalling.

The aims of the present study were to evaluate the GLP-1R (A316T) mutant receptor by:

a. Assessing changes in the cell surface expression of the mutant GLP-1 receptor using flow cytometry and live cell imaging.
b. Assessing changes in G-protein- and β-arrestin-dependent signalling properties caused by the mutant receptor using BRET-based biosensors to measure cAMP accumulation, intracellular Ca^{2+} mobilization and β-arrestin recruitment.
c. Assessing how these changes can lead to decreased T2D risk.

CHAPTER 1. Literature Review

1.1 The Incretin Effect

The term "incretin effect" is used to describe the fact that orally ingested glucose produces a much greater insulin response compared to the same amount of glucose administered intravenously (**Figure 1**). This phenomenon has been known since 1902, when Bayliss and Starling suggested that intestinal mucosa produced a factor (called secretin) in response to food ingestion, which stimulates the pancreas to secrete a substance, subsequently identified as insulin, which is capable of reducing blood glucose levels [19]. Thirty years later, La Barre proposed the name "incretin" for a substance extracted from the upper gut mucosa, and which produced hypoglycemia. While La Barre suggested that this substance could be the basis for diabetes therapy [20], the incretin concept was developed further only in the early 1960s when the measurement of insulin levels in blood became possible and the gut incretin peptides were identified [21-23].

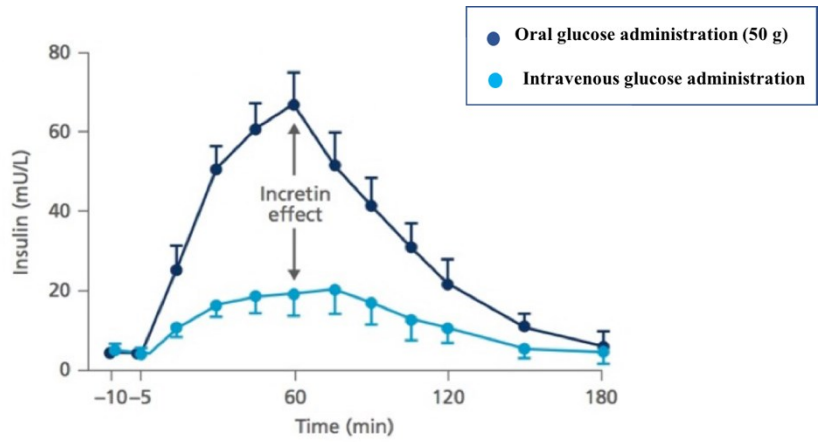

Figure 1. Insulin response to oral and intravenous glucose loading. Greater amounts of insulin are released in response to oral glucose administration compared to intravenous infusion of the same amount of glucose. This incretin effect results from the release of incretin peptides in the intestine following the food ingestion. Incretin hormones stimulate additional insulin release, beyond that produced by the direct effect of plasma glucose on pancreatic β-cells. Adapted from [24].

The incretin effect was explained by the release of the incretin hormones from the gut in response to food intake and their insulinotropic action in pancreatic β-cells [23, 25]. In total, the incretin effect accounts for more than 50% of the total insulin secreted after meals. Many hormones have been suggested to contribute to the incretin effect, but only two have been proved to be incretin hormones: glucose-dependent insulinotropic polypeptide (also known as gastric inhibitory peptide, GIP) [26, 27], and glucagon-like peptide-1 (GLP-1) [28]. Both incretin hormones are secreted by endocrine cells located in the epithelium of the small intestine. They have low basal concentrations in plasma and their release is regulated in a similar way to other digestive hormones. At low glucose concentrations (less than 4 mM) incretins are ineffective. An increase in the glucose concentration in the digestive tract acts as the trigger for hormone secretion by gut enteroendocrine cells, from where they are carried through the circulation to their target: the pancreatic β-cells (**Figure 2**). Stimulation of β-cells by incretins causes them to secrete more insulin. Several studies have shown that only the combined effects of GIP and GLP-1 account for the totality of the incretin effect [9, 29, 30].

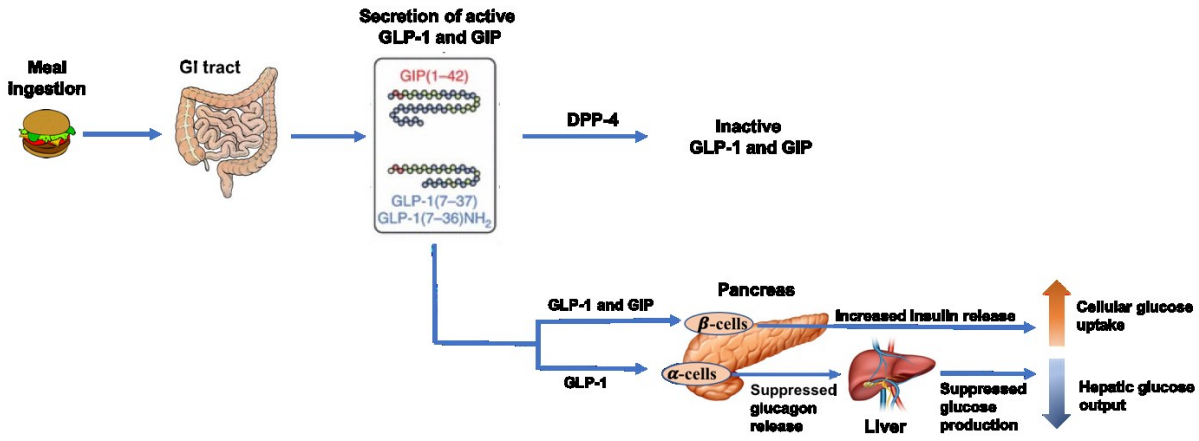

Figure 2. Insulin increasing action of incretin hormones. After a meal, nutrients in the small intestine stimulate GLP-1 and GIP secretion from L-cells in the ileum and colon. Active GLP-1 and GIP are carried through the blood circulation to their target: the pancreatic β-cells. Stimulation of β-cells by incretins causes them to secrete more insulin.

1.2 GLP-1 Expression

GLP-1 is a 30-amino acid peptide produced in the intestinal epithelial L-cells after differential processing of proglucagon, a 180-amino acid protein produced by the glucagon

gene (GCG) [31]. Proglucagon mRNA is expressed in three types of human cells: L-cells of the intestines, pancreatic α-cells and in some types of neurons [32], and is processed into different products through tissue-specific posttranslational processing (**Figure 3**). When the glucagon gene is expressed in pancreatic islets, the major product is glucagon. Studies of glucagon biosynthesis have also shown the presence of a large molecule, subsequently called the major proglucagon fragment (MPGF), which did not contain the glucagon peptide sequence and was formed in parallel with glucagon [33]. After proglucagon was cloned and sequenced in 1983, it became evident that MPGF contained two glucagon-like sequences which were attributed to GLP-1 and GLP-2 [34, 35]. This was confirmed by further studies showing that the two peptides stayed in the major proglucagon fragment when the glucagon gene is expressed in the pancreas (**Figure 3**). Proglucagon processing in the pancreatic α-cells is performed by the proprotein convertase 2 (PC2), which has been demonstrated to be necessary and sufficient for cleaving proglucagon. In the intestinal mucosa, however, proglucagon processing follows a different mechanism involving proprotein convertases PC1 and PC3; and results in the separate secretion of the two glucagon-like peptides [32].

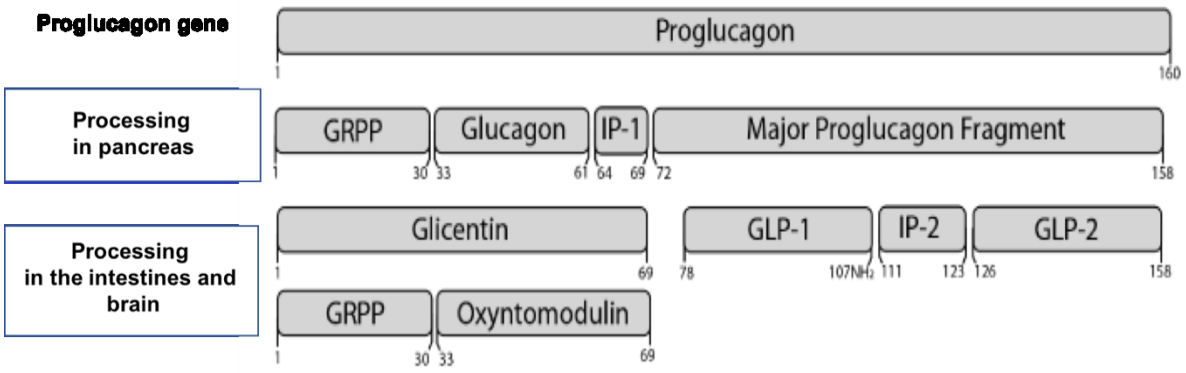

Figure 3. Posttranslational processing of proglucagon in different tissues. In the pancreas, processing of proglucagon leads to the generation of Glincentin-related polypeptide (GRPP), glucagon, intervening peptide-1 (IP-1) and the major proglucagon fragment. Glincentin, oxyntomodulin, intervening peptide-2 (IP-2), GLP-1, and GLP-2 are liberated after proglucagon processing in the intestine and brain. Adapted from [36].

The GLP-1 sequence begins with a histidine at the amino-terminus, as do most of the peptide hormones in the glucagon-related superfamily of hormones. Sequence analysis of

proglucagon showed that the first histidine is preceded by two basic amino acids, Lys-Arg, followed by four residues, another single basic residue, arginine, and a second histidine. Initially, it was believed that PC1/3 would cleave proglucagon during posttranslational processing in intestines at the Lys-Arg site, yielding a peptide of 37 or 36 amino acids, depending on whether glycine was present at the peptide C-terminal. If glycine was absent, the C-terminal arginine would be amidated, resulting in GLP-1 (1-36) amide. The proposed peptides were synthesized and, to the big surprise of the researchers, newly synthesized GLP-1 (1-36) amide and GLP-1 (1-37) did not show any insulinotropic activity [37]. Comparison of the GLP-1 sequence with the other members of glucagon family (**Figure 4**) led to the suggestion that GLP-1 may exist in a N-terminally truncated form as the peptide had better structure alignment with other glucagon hormones starting with histidine at the position 7, and not at position 1 as it was previously believed [35, 38]. In 1986, it was confirmed that GLP-1was indeed further N-terminally truncated by posttranslational processing in the intestinal L cells [39, 40]. In contrast to GLP-1(1-37), the truncated form of GLP-1(7-37) and GLP-1 (7-36) amide were found to be potent insulinotropic hormones in the isolated perfused pancreas of rats and pigs [41, 42].

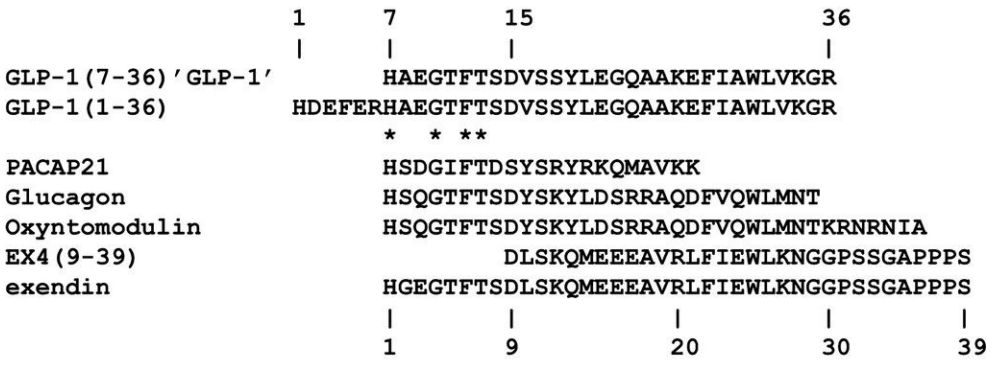

Figure 4. Aligned amino acid sequences of the peptides of glucagon family [43]. The sequences of the glucagon-related superfamily of hormones begin with a histidine as the amino-terminal residue. When the GLP-1 sequence was compared with the other members of glucagon family of peptide hormones, better alignment was observed for a peptide starting with histidine at the position 7, and not at position 1. This led to the suggestion that GLP-1 may exist in the N-terminally truncated form which was subsequently confirmed.

In humans, almost all GLP-1 is amidated (i.e. GLP-1 (7-36) amide), whereas many mammals also express the GLP-1 (7-37) form in lesser amounts [44]. While both forms have similar activities and metabolism [45], the amide form has slightly improved stability towards plasma enzymes [46]. When the GLP-1 sequence was compared among different species of mammals, complete preservation of the peptide structure was observed [47].

1.3 GLP-1 Secretion and Metabolism

GLP-1 secretion from L-cells is regulated by nutrient, neural and endocrine factors. Meal ingestion is the primary physiological stimulus for GLP-1 secretion and occurs in a biphasic manner [32]. The first phase of GLP-1 secretion takes place within 15-30 minutes after nutrient ingestion; and is followed by a prolonged second phase 90-120 minutes after food intake. Since most L-cells are located in the distal small intestine, the early phase of GLP-1 secretion is unlikely mediated by direct nutrient contact. Indeed, the autonomic nervous system has been suggested to mediate the cross-talk between the proximal and distal small intestine, with direct activation of the vagus nerve leading to increased meal-induced GLP-1 secretion [48]. Acetylcholine and gastric-releasing peptide have been proposed as the key neurotransmitters that mediate this cross-talk [49-51]; and muscarinic receptors have been demonstrated to play a crucial role in enhancing GLP-1 release [50].

In contrast, the second phase is believed to occur in response to direct interaction of the distal L-cells with luminal nutrients as they move down the small intestine. Interestingly, fat has been suggested to be the more important physiological regulator of GLP-1 secretion than glucose since glucose does not reach the distal gut in high concentrations [52]. Additionally, intestinal somatostatin, GABA, leptin and insulin also modulate GLP-1 release. Insulin resistance in the L-cells decreases both phases of GLP-1 secretion, an effect observed in both obese and T2D patients.

The half-life of GLP-1 in the circulation is very short (less than 2 minutes), due to the action of dipeptidyl peptidase IV (DDP-IV), which cleaves off the two N-terminal amino acids [53]. DPP-IV is a serine protease that specifically cleaves dipeptides from the amino termini of proteins and is widely present in blood and on cell membranes. The resulting GLP-1 cleavage peptides, GLP-1 (9–36) amide and GLP-1 (9–37), are inactive and their further role is not clear; some *in vitro* studies suggest that they may act as competitive antagonists at the GLP-1 receptor [54, 55], although this antagonism was not confirmed *in vivo* [56]. These metabolites

are cleared rapidly, mainly in the kidneys, with a half-life of 4–5 min [57, 58]. Owing to the rapid actions of DPP-IV, only 10–15% of newly secreted GLP-1 reaches the circulation in the intact form [59]. GLP-1 degradation can be prevented by using the inhibitors of DPP-IV, which are important clinical modulators of the GLP-1 pathway [53].

1.4 Physiological Roles of GLP-1

The actions of GLP-1 have been extensively studied over the last 20 years due to its ability to lower blood glucose level by increasing pancreatic insulin secretion after intravenous infusion or subcutaneous administration (**Figure 5**).

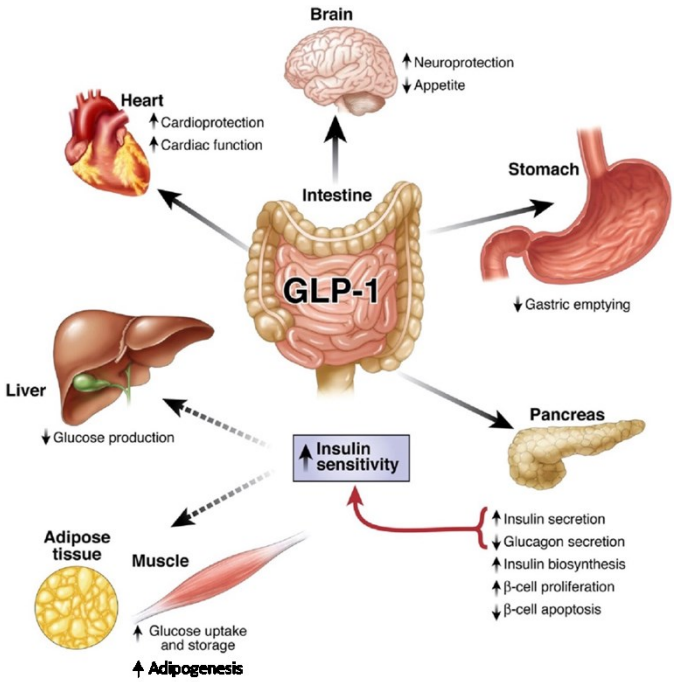

Figure 5. Versatile actions of GLP-1 in different organs and tissues. The main action of GLP-1 occurs at the pancreas where GLP-1 stimulates insulin secretion and inhibits glucagon production in a glucose dependent manner. In addition, GLP-1 also decreases appetite, slows gastric emptying, increases cardiac function and participates in other physiological actions as indicated in the diagram (Figure downloaded from [60]).

GLP-1 also mediates the reduction in blood glucose by suppressing glucagon secretion [61, 62]. Glucagon is a hormone whose effects oppose insulin action by increasing blood glucose levels by stimulating the breakdown of glycogen and gluconeogenesis in the liver. The

mechanism of GLP-1-induced inhibition of glucagon secretion is not completely elucidated [32]. It may result through effects on insulin release, which is generally thought to inhibit glucagon secretion; however, recent studies in type 1 diabetic patients without residual β-cell function showed a preserved and pronounced inhibitory effect of GLP-1 on glucagon secretion, what would suggest the possibility of other mechanisms [63]. Somatostatin secretion stimulated by GLP-1 might also play a role in inhibiting glucagon secretion through paracrine interactions [64]. The inhibitory effect of GLP-1 on glucagon secretion is seen *in vivo* only at levels of glucose that exceed normal fasting levels and disappears at levels just below normal fasting values [32]. Studies have shown that normal stimulation of glucagon secretion was not impeded by the GLP-1, suggesting that treatment with GLP-1 does not lead to an increased risk of hypoglycaemia [32, 65].

In addition, GLP-1 exerts non-insulinotropic actions that also contribute to glycemic control, including controlling pancreatic β-cell proliferation and survival. Studies in rats have shown that GLP-1 can increase the number of β-cells, either through promoting their growth or by inhibiting apoptosis. This hormone restores glucose sensitivity to previously glucose-resistant β-cells *via* up-regulation of the glucose transporters and glucokinase.

GLP-1 also has several extra-pancreatic functions. GLP-1 signalling in the central nervous system (CNS) exerts important regulatory effects on feeding behavior, blood glucose levels and cardiovascular functions [9]. In the nervous system GLP-1 has been shown to stimulate its cognate receptor GLP-1R in the brain and is responsible for signalling satiety and thus inhibiting feeding which results in the weight loss. In the gastrointestinal tract, GLP-1 was also found to inhibit gastrin-induced acid secretion, gastric emptying and gastric motility to slow the appearance of exogenous glucose in the blood [66]. Attenuation of gastric emptying is important for normalizing postprandial blood glucose levels especially in T2D patients when the transit of nutrients from stomach to small intestines is reduced [32]. The mechanism of GLP-1 action in the gut is highly complex and may also involve GLP-1 signalling in the autonomic nervous system through a "gut-brain-periphery" pathway [9, 67].

There is also growing evidence for GLP-1 signalling in adipose tissue, where the level of GLP-1R expression has been shown to correlate with insulin resistance [68, 69]. In adipocytes GLP-1 regulates adipogenesis and restores insulin sensitivity.

In the cardiovascular system GLP-1R has been demonstrated to improve endothelial and cardiac function [70-72]. GLP-1R is expressed in the heart and GLP-1 was shown to increase

cAMP levels in adult rat cardiac myocytes [32]. Mice lacking GLP-1R at the age of 2 months had reduced resting heart rate and elevated left ventricular end-diastolic pressure, compared to controls [32]. The ability of GLP-1 to significantly reduce infarction size in both *in vitro* and *in vivo* models of ischemia-reperfusion damage has been demonstrated recently [73]. There are also studies describing the expression of GLP-1R in the lungs [74], although the functions of GLP-1 are not yet completely understood, this hormone may play the role in increasing the secretion of macromolecules from the neuroendocrine cells [32].

1.5 GLP-1R Structure and Activation

GLP-1 exerts its action through the GLP-1 receptor, GLP-1R, a 463 amino acid-long protein that is mainly expressed in pancreatic β-cells. GLP-1R mRNA can be identified in other tissues, including the lung, stomach, kidney, hypothalamus, brain, adipose, and heart, but not in skeletal muscle, and liver. The cDNA of GLP-1R was first isolated using a rat pancreatic islet cDNA library; and its function as a receptor for GLP-1 was confirmed by binding experiments with radiolabeled GLP-1 [75]. Subsequently, the human GLP-1R cDNA was isolated and was shown to share 90% homology with the rat receptor at the amino acid level [76]. The human GPL-1R gene is located on chromosome 6 and contains 13 exons [77].

GLP-1R is a member of GPCR family B, which includes a number of receptors for classical peptide hormones, such as neuropeptides (Corticotropin-releasing hormone and Growth hormone releasing hormone receptors), hormones regulating calcium levels (Calcitonin and Parathyroid hormone receptors) and gut peptides (Glucagon, Gastric inhibitory polypeptide, Secretin and Vasoactive intestinal peptide receptors). GPCR Family B (also called GPCR Family 2 or the secretin-like family) includes 15 members and is characterized by a large N-terminal domain (~ 100-160 amino acids in length) that is stabilized by the three conserved disulfide bonds. Like other members of the family, GLP-1R contains a large hydrophilic N-terminal extracellular domain with a putative signal peptide, seven hydrophobic transmembrane domains (TM1-TM7) joined by three hydrophilic intracellular (ICL1-3) and three extracellular (ECL1-3) domains with an intracellular C-terminus (**Figure 6**). Ligands for receptors in this family are large peptides with paracrine activities. By sequence similarity, GLP-1R forms a subgroup with GLP-2R, GCGR, (the glucagon receptor), and GIPR (the GIP receptor) [78]. These receptors play an essential role in maintaining glucose homeostasis.

Structural data on GPCR family B is much more limited than that on GPCR family A; and there are only experimentally determined structures of the N-terminal extracellular domains of a few receptors, including GLP-1R and GIPR [79, 80]. These structures reveal important information about mechanisms of the interaction between the receptors and their ligands. The published data has shown that the receptor N-terminal domain contains conserved three-layered β-α-β fold, forming two anti-parallel β-sheets. This structure is stabilized by three interlayer disulfide bonds formed by six conserved cysteine residues (**Figure 6**). Additional

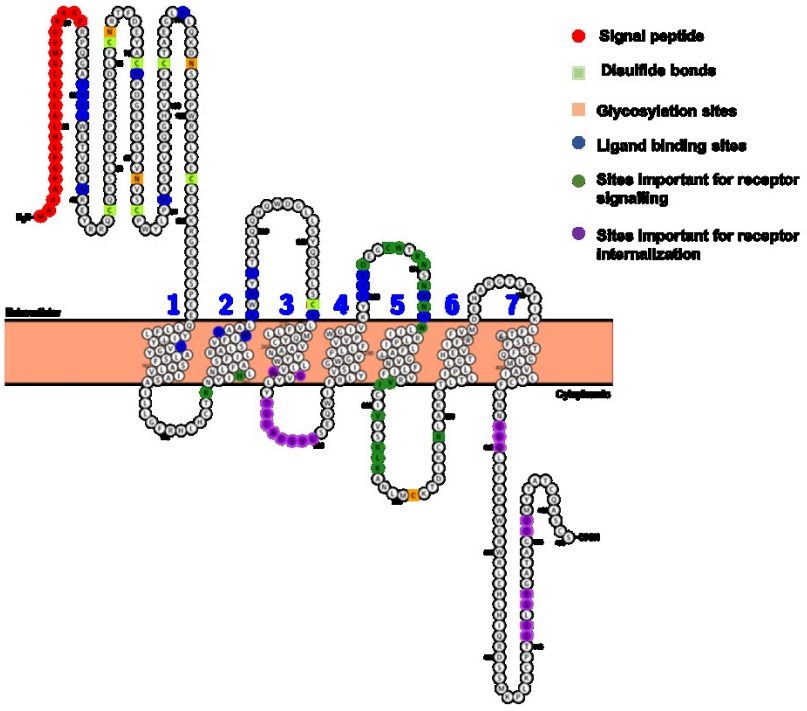

Figure 6. Amino acid sequence of hGLP-1R showing main functional domains. A structure plot of GLP-1R was created using the Protter software [81] and has been colored to highlight the location of signal peptide, glycosylation and phosphorylation sites, disulfide bonds, and residues important for ligand binding and receptor signalling.

hydrophobic packing interactions and hydrogen bonds help to stabilize the second and third layers. Five conserved residues Asp^{113}, Trp^{118}, Pro^{132}, Gly^{152} and Trp^{154} are suggested to play an important role in these interactions [82, 83]. In addition, the GLP-1R structure suggests that a fourth disulfide bond is formed by residue Cys^{226} and Cys^{296}, where Cys^{296} is in the third extracellular helix and probably is close to the activation pocket as a mutation of Cys^{226} to alanine decreases GLP-1 potency [84]. It has been proposed that the N-terminal eight

amino acids of GLP-1 bind to the receptor transmembrane domain, including connecting loops leading to the receptor activation [85, 86]. The C-terminal 22 amino acids of GLP-1 are thought to bind to the large N-terminal domain of the GLP-1R, and the most binding energy originates from this interaction.

The structure of the transmembrane and intracellular domains of GLP-1R is unknown and believed to be similar to family A GPCRs [87]. Intracellular loop 3 (IC3) has been shown to be critical for GPCR interaction with G proteins for a number of receptors [88]. Studies on GLP-1R have shown the importance of Lys^{334}-Leu^{335}-Lys^{336} residues in the IC3 in mediating G-protein coupling and activation [89] (**Figure 6**). This finding was supported by the fact that the IC3 sequences of GLP-1R, GIPR and GCGR have the only conservative KL motif in their IC3 domain and a correct positioning of charged amino acids was suggested to play an important role in G protein interaction [89-91]. Moreover, studies have shown that the deletion of the Lys^{334}-Leu^{335}-Lys^{336} sequence in GLP-1R abolishes the activation of the adenylyl cyclase and subsequently, insulin secretion.

It is not completely known whether the IC3 loop junction directly activates the G protein or merely serves as a domain controlling the orientation of the IC3 loop and thus the interaction of the receptor with G proteins. The residues V^{327}, I^{328}, and V^{331} in the GLP-1R IC3 loop also have been shown to be essential in interaction with G protein and stimulation of cAMP production. It was suggested that these residues form hydrophobic face which couples directly with G protein [92].

A signal peptide at the extreme N-terminus of the GLP-1R was shown to be required for the receptor's synthesis and trafficking in HEK-293 cells [93]. The authors suggested that cleavage of the signal peptide was essential for correct GLP-1R processing and trafficking, such that only the mature receptor reached the plasma membrane [93]. It has been also shown that GLP-1R is a glycoprotein, and that glycosylation, together with the cleavage of the signal peptide, is an essential process in the correct processing of the receptor [93-95].

1.6 GLP-1R Signalling

Following GLP-1 binding, GLP-1R undergoes a conformational change which allows binding of the G-protein complex. GLP-1R signalling has been most extensively studied in pancreatic islet β- cells where it leads to insulin synthesis and secretion and also enhances

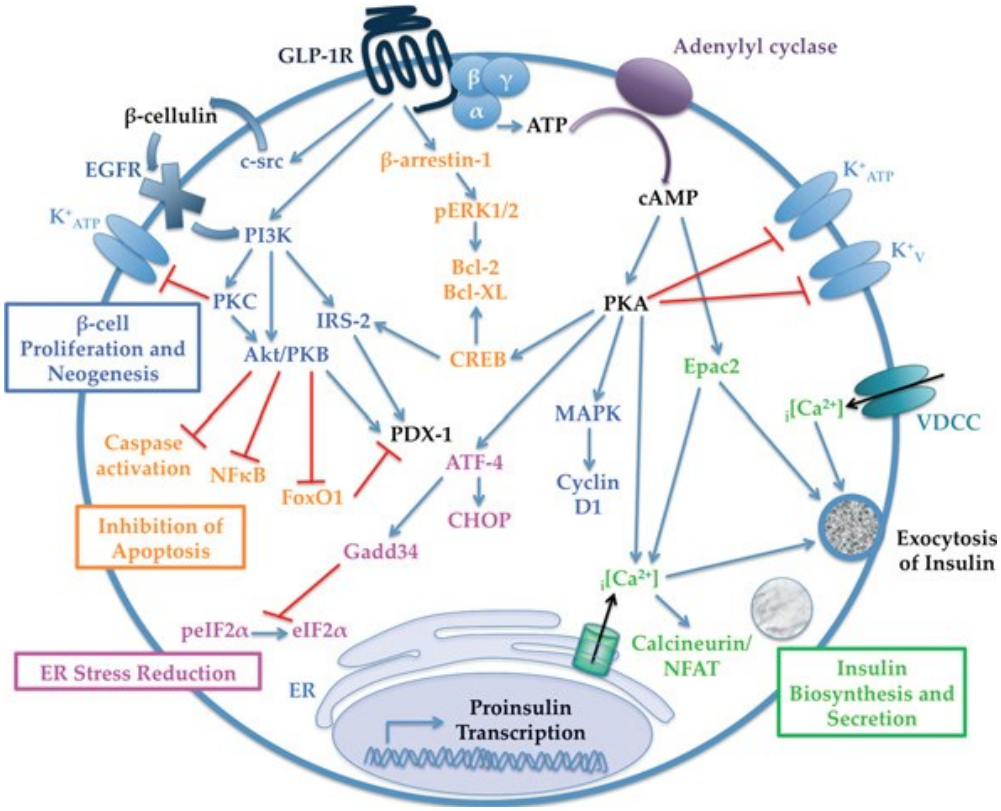

Figure 7. Major signalling pathways activated in response to GLP-1R activation in β-cells [96]. GLP-1R activation has major downstream effects on acute insulin synthesis, insulin secretion, preservation of β-cell mass and function. Signalling in pancreatic β-cells *via* GLP-1R coupling to $G\alpha_s$ mediates an increase in cAMP that up-regulates PKA and EPAC2 activity. These pathways increase intracellular Ca concentration by 1) inhibiting K^+ channels and 2) accelerating Ca2+ influx through voltage-gated Ca2+ channels (VGCCs). Together these effects lead to increased insulin biosynthesis and secretion. Activation of the proto-oncogene tyrosine kinase src (c-src) and subsequent transactivation of the epidermal growth factor receptor (EGFR) result in increased PI3K, IRS-2 (insulin receptor substrate 2) and PKB activity to enhance β-cell proliferation. This is also facilitated in part by PKA-mediated increases in mitogen-activated protein kinases (MAPKs), β-catenin and cyclin D1. Inhibition of 1) caspases, 2) forkhead box protein O1 (FoxO1), and 3) nuclear factor κB (NF-κB), in addition to 4) upregulation of the cAMP-response-element-binding protein (CREB) and 5) subsequent inhibition of Bcl2-associated death promoter (BAD) inhibit apoptosis, a process also mediated by β-arrestins and pERK1/2.

β-cell function (**Figure 7**) [97]. Predominantly, GLP-1R in β-cells binds $G\alpha_s$ proteins, resulting in the activation of membrane-bound adenylyl cyclase (AC), which in turn catalyzes the conversion of ATP into cAMP, the main mediator GLP-1 action in β-cells [98]. The increase in cAMP levels leads to up-regulation of protein kinase A (PKA) activity [99, 100]. PKA is a ubiquitous serine/threonine protein kinase and is a key component in the regulation of insulin secretion by cAMP. Studies by Wang [100] showed that inhibition of PKA in isolated islets and insulinoma cell lines diminishes GLP-1 mediated insulin secretion.

Increased cAMP levels in β-cells in response to GLP-1 stimulation activates both PKA and Epac2, but the precise mechanism by which each effector contributes to overall insulin secretion is still uncertain [101]. Studies of the mechanism of EPAC action showed that their cAMP binding sites have much lower affinity for this cyclic nucleoside compared to PKA. Recently it has been shown that localized low concentrations of cAMP lead to preferential activation of PKA [101]. Thus, it is probable that EPAC is sensitive to cAMP in a range where PKA is already saturated, which is important when considering the physiological relevance of the pathways in the regulation of insulin secretion and β-cell cycle.

After activation, PKA is believed to directly inhibit K_{ATP} channels by the phosphorylation of its sulfonylurea receptor (SUR1) subunit, which causes depolarization of the plasma membrane and leads to the opening of voltage-gated Ca^{2+} channels (VGCC) increasing intracellular Ca^{2+} as a result of Ca^{2+} influx through these channels. Epac2 is known to directly promote mobilisation of Ca^{2+} from intracellular Ca^{2+} stores, which in turns triggers calcium-induced Ca^{2+} release (CICR) [102-104]. This overall increase in cytosolic Ca^{2+} ($[Ca^{2+}]_I$) triggers fusion of insulin-containing secretory vesicles to the plasma membrane, and exocytosis of insulin follows rapidly. Therefore, the combination of both cAMP production and influx of Ca^{2+} are vital components in the secretion of insulin.

The process of insulin exocytosis has been studied extensively in various β-cell systems [105]. There are three functionally different pools of insulin secretory vesicles in β-cells, the reserve pool (RP) located deep in the cytoplasm, the readily releasable pool (RRP) and the immediately releasable pool (IRP), both located close to the membrane [106, 107]. It is widely known that glucose-stimulated insulin secretion from pancreatic β-cells occurs in two phases (**Figure 8**) [108]. The first phase (also called the triggering phase [109]) is rapid, reaches a peak within 10 min in the mouse, and lasts for about 10-15 min. Termination of the first phase in humans is designated as a nadir and determines the complete depletion of the

IRP. When recruitment from the RP begins, insulin secretion gradually increases starting the second phase (also called the amplifying phase) until it reaches a plateau which lasts for the duration of glucose stimulation [106]. The rate-limiting step in the second phase of insulin secretion is the mobilization of vesicles from the RP to the RRP and subsequently to the IRP. The underlying mechanisms for the amplifying phase are still unknown.

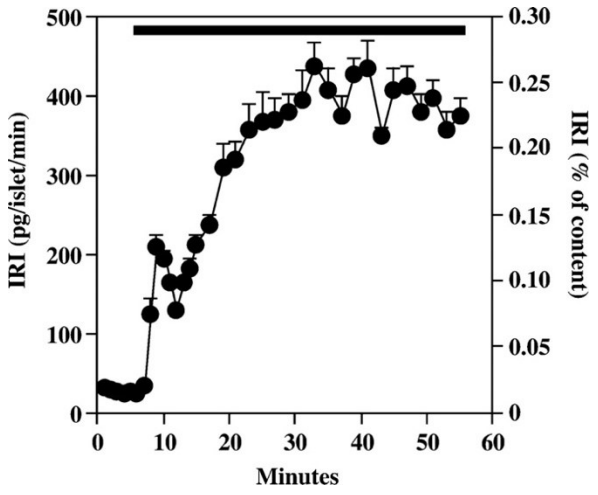

Figure 8. Glucose-stimulated biphasic insulin release in rat islets [106]. The first phase of insulin secretion is rapid, and reaches a peak within 10 minutes in the mouse and lasts for about 10-15 minutes. When recruitment from the reserve pool (RP) begins, insulin secretion gradually increases starting the second phase which lasts for the duration of glucose stimulation.

There are two basic and opposing theories regarding relevant contribution of Epac2 and PKA to the triggering phase of insulin secretion after GLP-1R activation. According to the first theory, EPAC is solely responsible for the rapid increase in intracellular Ca^{2+} and insulin exocytosis [110]. The second theory supports the importance of residual PKA levels to prime the rapid insulin release [108]. Recent findings, however, support the idea of the equal involvement of both effectors [111, 112].

Sustained oscillatory increases in cAMP by GLP-1R activation also results in translocation of PKA to the nucleus [113, 114] where it has been shown to be directly involved in increasing insulin transcription through stabilization of the insulin transcript. In 1987, Drucker et al. first demonstrated the ability of GLP-1 to increase insulin mRNA levels. Later studies have proved that PKA phosphorylates Ser^{133} of the cAMP response element-binding protein

(CREB) in the nucleus, which induces conformational change in CREB and permits it to bind to the transcriptional coactivator CREB binding protein (CBP) [115] (**Figure 7**). The resulting complex, bound to cAMP response elements (CREs) located at 5' gene promoters, stimulates expression of the insulin and insulin receptor substrate-2 (IRS-2) genes [116-118]. PKA-mediated induction of IRS-2 expression also plays a role in promoting β-cell growth. PKA also activates nuclear translocation of the transcription factor PDX-1, which plays a key role in β-cell and pancreatic development and in regulating insulin gene expression [108].

GLP-1R also mediates activation of the epidermal growth factor receptor (EGFR) by enhancing c-src and subsequent β-cellulin production, which results in promotion of phosphoinositide-3-kinase activity and nuclear translocation of protein kinase C-ξ and transcription factors which contribute to β-cell proliferation [119].

The role of alternate $G\alpha_q$ subunits in mediating GLP-1R signalling has not been completely elucidated. Usually signalling through $G\alpha_q$ is believed to activate phospholipase C (PLC), which in turn hydrolyses phosphatydilinositol-4,5-biphosphate (PIP_2) to inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) [120]. IP_3 binding to the IP_3 receptor (IP_3R) in the endoplasmic reticulum (ER) leads to increased cytoplasmic Ca^{2+} levels, which cause the activation of PKC and the subsequent induction of extracellular signal-regulated kinase (ERK) phosphorylation [121, 122]. GLP-1 has been shown to activate ERK and p38MAP kinase but whether it acts downstream of $G\alpha_q$ or $G\alpha_s$ is disputed [6, 123]. A recent study by Thompson [124] suggested an important role for $G\alpha_q$ in the agonist-induced receptor internalization through activation of PKC.

In addition to signalling *via* G proteins, recently emerging studies suggested that GRK (GPCR kinase) and β-arrestin recruitment are also involved in GLP-1R downstream action [7, 8, 125, 126]. β-Arrestin1 knockdown in β-cells resulted in attenuated GLP-1 signalling, leading to decreased insulin receptor substrate expression, cAMP production and insulin secretion [7]. A recent study by Quoyer [8] has suggested that two different distinct pathways of ERK1/2 phosphorylation (pERK1/2) alter GLP-1R activation. The first is a PKA-dependent pathway that mediates rapid and transient pERK1/2 activation followed by nuclear translocation of the activated kinase. The second involves late pERK1/2 activation by β-arrestin-1 and is restricted to the β-cell cytoplasm. The same authors have shown that β-arrestin-1-dependent ERK1/2 signalling stimulates p90RSK activity by mediation of Bcl-2 phosphorylation, which leads to an anti-apoptotic effect of GLP-1R [8].

1.7 GLP-1 Induced Receptor Internalization

Following stimulation with an agonist, GPCRs are rapidly desensitized through phosphorylation by G protein-coupled receptor kinases (GRKs) [127]. This facilitates the recruitment of intracellular scaffolding proteins such as β-arrestin and promotes receptor uncoupling from the G protein and its translocation to the endocytic machinery *via* rapid internalization [128]. Once internalized, receptors may be down-regulated via lysosomal degradation or recycled back to cell membrane. β-Arrestins are known to play an important regulatory role in these processes. GPCRs that display strong and persistent β-arrestin binding recycle relatively slowly and are more likely to be transported to the lysosomes for the receptor degradation [129]. In contrast, if β-arrestin dissociates from GPCRs shortly after movement of the receptors into the clathrin-coated vesicles, recycling will be quite rapid. In recent years the interaction between GPCRs and β-arrestins has received increased attention as it became evident that this interaction is able not only to terminate receptor G protein coupling but also to initiate alternative signalling pathways [130].

Both the rat and the human GLP-1R have been shown to internalize rapidly and recycle back to the cell membrane after activation by GLP-1 and other agonists [125, 131-133]; although there is currently some controversy about the pathways by which the GLP-1R is internalized [36]. It has been reported that receptor internalization is mediated by clathrin-coated vesicles and that three phosphorylation sites within the receptor C-terminus play an important role in this process [132]. However, a recent study has shown that the GLP-1R is internalized by a caveolae-mediated pathway which is independent of β-arrestin-mediated events [134].

1.8 GLP-1 and GLP-1R as Therapeutic Targets for Type 2 Diabetes

Major clinical interest in GLP-1 is based on its role in the development and treatment of T2D. T2D is characterized by a severely reduced or even absent incretin effect, resulting in inappropriate insulin secretion [24]. It is now clear that the lack of incretin effect is due to the complete loss of the insulinotropic action of GIP [135, 136], whereas the secretion of GLP-1 is normal or almost normal [32]. In addition, dose-response studies have shown that the potency of GLP-1 with respect to enhancing glucose-induced insulin secretion is greatly reduced (to 20%) in T2D patients compared with non-diabetic controls [137].

The molecular mechanisms that lead to the reduced β-cell sensitivity to GLP-1 and the nearly absent sensitivity to GIP are currently unknown [135]. In this context, the fact that insulin secretion can be restored to normal levels by administration of GLP-1 has attracted clinical interest in using GLP-1R agonists to treat T2D. Therefore, therapeutic strategies based on enhancing GLP-1 action and activating GLP-1R on β-cells have been extensively developed. One of the major drawbacks to the use of the natural ligand in the clinic for treating T2D is its rapid degradation *in vivo* by DPP-IV [138]. Many modifications have been made to GLP-1 to increase its half-life in the plasma and its efficacy in humans [139].

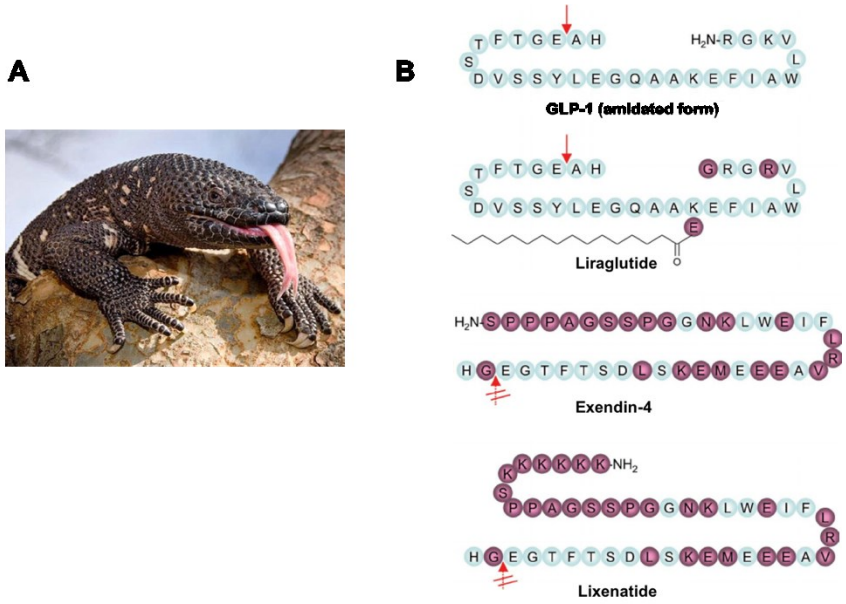

Figure 9. GLP-1R agonists. A. Gila monster, from whose salivary glands of the hormone exendin-4 was extracted. **B.** Structures of native GLP-1, liraglutide, exendin-4, and lixenatide. Histidine is the N-terminal amino acid in all analogs. Amino acid modifications are highlighted in purple. The DPP-IV cleavage site is indicated with red arrows. Red letters indicate changes from GLP-1 introduced in derivatives or that occur naturally in exendin-4.

All other GLP-1 mimetics have been developed synthetically, based on designs that are usually directed to enhance the compound's stability and/or function *in vivo*. Although N-terminal modifications of native GLP-1 can prevent DPP-IV degradation, gradual renal elimination of the modified peptide still limits their biological activity to approximately 4h [140]. Therefore, further modifications at the C-terminus are used to extend the biological

half-life of GLP-1R agonists beyond a few hours. Fatty acid derivatization of target peptides has also been successfully employed to extend their duration of action.

The best-known GLP-1 mimetic prescribed for T2D is exenatide (Byetta®), a synthetically produced equivalent of exendin-4 (Exe-4). This peptide is extracted from salivary glands of the *Heloderma suspectum* or Gila monster lizard, native to Gila county in southern Arizona [108] (**Figure 9A**). Like GLP-1, Exe-4 administration can quickly decrease plasma glucose levels following nutrient ingestion in both healthy and diabetic subjects through multiple mechanisms, including enhancement of glucose-dependent insulin secretion, suppression of excess glucagon secretion, reduction of food intake, and slowing of gastric emptying [33]. However, Exe-4 does not have the same DPP-IV cleavage site and therefore has prolonged activity *in vivo* due to its stability to the proteolytic activity of this enzyme (**Figure 9B**). Exe-4 is cleared in the kidneys by glomerular filtration which increases a half-life of the molecule to 30 minutes and requires twice-daily injection [32].

Liraglutide (also known as Victoza®, Novo Nordisk), was synthesized by covalently coupling a hexadecanoic fatty acid to the Lys^{26} residue of GLP-1 while Lys^{33} was substituted with arginine to prevent acylation here [141] (**Figure 9B**). Like GLP-1 and exendin-4, liraglutide significantly improves glycaemic control, enhances β-cell function and promotes weight loss. Addition of albumin moiety has acquired some special pharmacokinetic properties to the molecule resulting in prevention of renal elimination of the bound molecule and subsequent increase of its half-life after subcutaneous injections to 12 h [142]. Clinically, the molecule administered as a once daily injection has been shown to have similar actions as continuously infused GLP-1; and is now widely prescribed to treat T2D [143].

The next generation of GLP-1R agonists include exenatide-long acting release (LAR) [144], taspoglutide [145], albiglutide [146], and CJC-1134-PC [147]. All these mimetics display prolonged action requiring once-weekly to once-monthly injection. Modification of GLP-1 and exendin-4 peptides through biotinylation and polyethylene glycol conjugation was used to improve oral delivery of antidiabetic treatments by enhancing intestinal absorption [148].

GLP-1R agonists are shown to exhibit cardiovascular benefits, such as lowering blood pressure, improving lipid profiles, and possibly even enhancing cardiac contractility and endothelial function [149].

Unfortunately, several concerns have been raised regarding the side effects of GLP-1-based therapeutics. Mild to moderate nausea, vomiting and diarrhea are often associated with incretin therapy [150]. Exenatide needs to be injected subcutaneously twice daily, which causes discomfort to patients and sometimes leads to withdrawal from treatment. Liraglutide is suitable for once-weekly administration, however, gastrointestinal side effects are common for this compound in addition to other concerns like weakness, confusion, fever [150].

Lately, a much bigger controversy has arisen over the long-term consequences of GLP-1-based therapies, including reports of pancreatitis and C-cell hyperplasia which may impose an increased risk of pancreatic and thyroid cancer due to GLP-1 proliferative effects in the pancreas and thyroid [150]. In a recent study, Nauck et al. [151] assessed the risk of acute pancreatitis during treatment with dulaglutide, a once-weekly GLP-1R agonist. Their results did not provide any evidence of the increased risk of pancreatitis event in patients treated with dulaglutide. The study was limited by small sample size and quite short duration of exposure which prevented it from making a definitive conclusion on the effects of long-term exposure to GLP-1R agonists on the exocrine pancreas. Additionally, some GLP-1 mimetics have shown less positive clinical outcomes: for example, taspoglutide, which has a very similar primary sequence to GLP-1, was withdrawn from clinical trials due to a high incidence of severe nausea, vomiting and systemic allergic reactions [18]. This effect may be attributed to differential activation and signalling at the GLP-1R.

DPP-IV inhibitors offer another alternative to traditional T2D treatment. These agents enhance GLP-1 activity by inhibiting the DPP-IV enzyme., but are less effective than GLP-1R agonists. [152]. One advantage of using DPP-IV inhibitors is the availability of oral tablets (current GLP-1R agonists require injections because they are not available in tablet form). There are currently three FDA-approved DPP-IV inhibitors: sitagliptin, saxagliptin and linagliptin. In the European Union, a forth, vildagliptin is also available. In contrast to GLP-1 mimetics, DPP-IV inhibitors do not have an effect on body weight, which possibly reflects the limited increase in GLP-1R stimulation observed during the treatment. Usually, sitagliptin and vildagliptin are more effective when they were used in combination with metformin [153]. In addition, DPP-IV inhibitors could also be used with insulin therapy. [154]. Because of wide range of action of DPP-IV inhibitors (on chemokines, hormones, and neuropeptides), side effects of long-term inhibition are not known. Considering superior glycaemic activity of GLP-1R agonists over DPP-IV inhibitors, they are often recommended over DPP-IV inhibitors, particularly if the patient is overweight [152].

1.9 Ligand-Directed Signalling Bias

Recent studies on the effect of multiple GLP-1R ligands on the downstream pathway have shown that they can promote different signalling profile after receptor activation. This phenomenon is called biased agonism and it is explained by the ability of ligands to stabilize different repertoires of receptor conformations [155]. Biased agonism at the GLP-1R has been reported by multiple groups and has substantial implications for development of therapeutic agents targeting this receptor [10, 11, 156, 157].

While GLP-1R ligands are known to activate primarily cAMP-dependent pathways, clinical outcomes may differ for different therapeutic compounds as a result of their effects on other downstream pathways. For example, oxyntomodulin and Exe-4 show reversed potency in β-arrestin recruitment and Ca^{2+} mobilization compared endogenous GLP-1 [11]. Interestingly, GLP-1(9-36)NH_2, the principal metabolite of GLP-1, is also a biased agonist that can promote ERK1/2 phosphorylation despite showing severely impaired potency for cAMP production and insulin secretion [156, 158]. In addition, GLP-1R internalization and recycling mediated by different ligands can also contribute to a biased agonist profile. GLP-1 and Exe-4 induce internalization of the receptor with 10-fold higher potency than Liraglutide, but recycling with Exe-4 and liraglutide is two-three times slower than observed with GLP-1 [133]. Recent studies indicate that GLP-1R can continue signalling after internalization inside the cell promoting distinct physiological functions [131]. This raises the possibility that the ability of different ligands to promote different kinetics of internalization and recycling of the GLP-1R may play an important role in the observed ligand-biased agonism.

1.10 Small molecule GLP-1R agonists

Historically, the discovery of non-peptide agonists of GLP-1R that may be suitable for developing orally available drugs has been generally unsuccessful [159]. Interaction of endogenous peptide hormones with their receptors typically involves large regions of the receptor, including specific ligand binding sites along with the receptor N-terminal extracellular domains [160]. Hence, the development of small molecules that can act at sites in GLP-1R that are distinct from the endogenous ligand binding sites (known as allosteric agonists) is highly desirable. Ligands acting allosterically have some major advantages from a therapeutic point of view. Their ability to simultaneously bind to the receptor at the same

time as the endogenous ligand can induce a new repertoire of receptor conformations which influence receptor activity; and their potential for oral administration make them highly attractive targets in drug development [161]. Allosteric ligands that enhance insulinotropic effects of the GLP-1 activated GLP-1R system (positive allosteric modulators, PAM) are being extensively studied.

A small number of allosterically acting ligands have been identified for GLP-1R (**Figure 10**) [161]. They represent different classes of compounds, including **1)** a series of quinoxalines, the best characterized being Compound 2 (6,7-dichloro-2-methylsulfonyl-3-tert-butylaminoquinoxaline) (Novo Nordisk) [162, 163]; **2)** modified pyrimidines, such as BETP or compound B (4-(3-(benzyloxy)phenyl)-2-(ethylsulfinyl)-6-(trifluoromethyl)pyrimidine) (Eli Lilly) [164]; **3)** substituted cyclobutanes such as Boc5 (1,3-bis[[4-(tert-butoxy-carbonylamino)benzoyl]amino]-2,4-bis[3-methoxy-4-(thiophene-2-carbonyloxy)-phenyl]cyclobutane-1,3-dicarboxylic acid) [165], and **4)** a series of compounds reported in patents by Transtech Pharma such as TT15 [166].

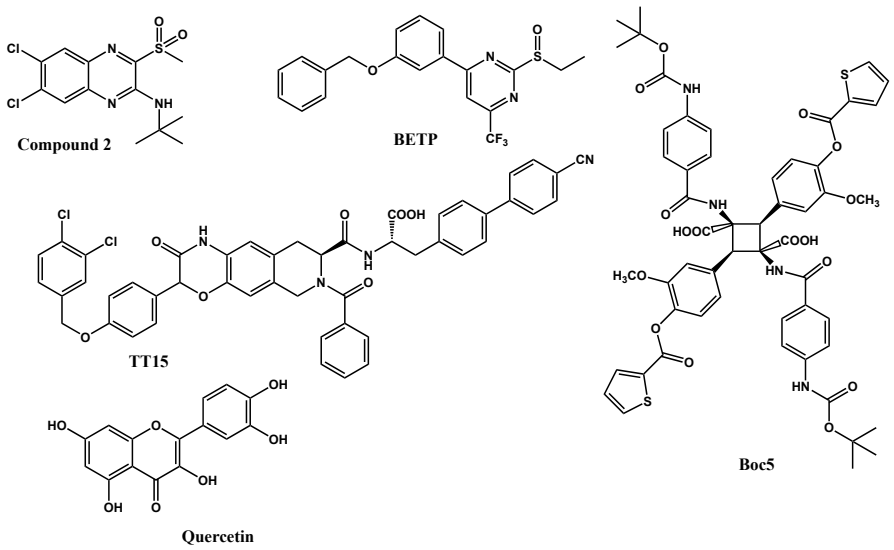

Figure 10. Structure of small molecule ligands of GLP-1R. Structural diagrams are shown for **Compound 2**, a synthetic allosteric agonist and positive modulator of cAMP formation; **BETP**, a synthetic allosteric agonist and positive modulator of cAMP formation; **TT15**, a synthetic allosteric agonist. Boc5, synthetic allosteric agonist; and **Quercetin**, a naturally occurring positive allosteric modulator of intracellular Ca^{2+} mobilization

Compound 2 was the first non-peptide agonist identified for GLP-1R that demonstrated glucose-dependent insulin release *via* the GLP-1R activation. Similarly, Boc5 was shown to be a fully efficacious agonist with the possibility to decrease plasma glucose levels and reduce nutrient uptake in obese mice with an effect comparable to the native GLP-1 peptide [166]. BETP, the most recently identified allosterically acting synthetic ligand increases glucose-dependent insulin release from normal and diabetic human islet: removal of the GLP-1R N-terminus does not influence the activity of this compound confirming the allosteric mode of action [159]. Additionally, all four allosteric ligands have been shown to display a significant signalling bias when compared with the endogenous peptides [10]. The most widely characterized ligands (Compound 2 and BETP) have an enhanced ability to recruit β-arrestins relative to cAMP when compared with the GLP-1 peptide, and also show some distinct effects on ERK phosphorylation and Ca^{2+} mobilization. The ability of allosteric ligands to induce substantially different signalling profiles than peptide ligands is related to their different mode of interaction with the receptor. In contrast to peptide ligands that bind the receptor at the extracellular surface, both Compound 2 and BETP interact with GLP-1R at the intracellular face *via* covalent binding at the third intracellular loop [167]. These findings have indicated that small allosteric ligands cannot fully mimic the action of large peptide hormones and more detailed studies are required in order to fully understand how these signalling profiles affect the synthesis and release of insulin along with the modulation of proliferation and apoptosis [96].

In addition to activating GLP-1R specifically and producing physiologically important responses, the allosteric GLP-1R ligands have been shown to differentially modulate peptide ligand actions [10]. For example, Compound 2 exhibits classic probe-dependent interactions with orthosteric peptide agonists that preferentially enhance oxyntomodulin action compared to other ligands [168]. In addition to this probe-dependent effect, Compound 2 altered the signalling profile of oxyntomodulin preferentially increasing cAMP pathway without any modulation of pERK1/2 or Ca^{2+} mobilization [96]. BETP also displayed strong probe-dependent, signal-biased interactions with orthosteric ligands, preferentially increasing cAMP production in response to oxyntomodulin and showing no effect on GLP-1(7-36)NH_2 and Exe-4 signalling [10].

Recent studies have shown that quercetin can allosterically modulate GLP-1R, leading to enhanced intracellular Ca^{2+} mobilization *in vitro* [11]. Unlike all synthetic allosteric ligands, quercetin is a naturally occurring compound, belonging to the flavonoid family, with a large

therapeutic potential. Flavonoids are known for their antibacterial, hepatoprotective, anti-inflammatory, anticancer, and antiviral activity [169]. Interestingly, quercetin also showed strong probe dependence and altered signalling bias while tested in β-cells. Studies have shown that quercetin lacked intrinsic efficacy in all GLP-1R-mediated signalling pathways assessed, but selectively augmented the efficacy in iCa^{2+} mobilization of the high-affinity agonists GLP-1(7–36)NH_2, GLP-1 (7–37), and Exe-4 [170].

All these data suggest that cobinding of both orthosteric and allosteric ligands can alter the GLP-1R conformation, resulting in changes in signalling profiles of the receptor. The differential cooperativity in binding and signalling for these small molecule ligands shows that the action of allosteric modulators cannot be classified by measuring one pathway. Additionally, it remains to be elucidated how biased signalling could be used clinically to enhance GLP-1R therapies.

1.11 GLP-1 Interacting Proteins and their Role in Receptor Signalling

To date, a limited number of interacting molecules that regulate GLP-1R function have been identified; these include the scaffolding proteins, β-arrestin-1 and caveolin-1, both known to play an important role in GLP-1R signalling [8, 134]. For example, the interaction between caveolin-1 and GLP-1R has been shown to stabilize receptor localization at the plasma membrane; and caveolin-1 deletion completely abolished GLP-1 binding and activity *in vitro* [134]. Another accessory protein, the Small ubiquitin-related modifier protein (SUMO), interacts with GLP-1R to down-regulate receptor trafficking to the plasma membrane and reduce GLP-1 signalling [171].

In addition, there is accumulating data showing that family B GPCRs can undergo both homo-and heterodimerization [172-174]. Recent studies have provided evidence that GPCR family B receptor dimerization is commonly associated with the presence of two lipid-exposed transmembrane TM-4 domains at the site of receptor-receptor interaction [172]. Disruption of this interface *via* mutation or co-incubation with TM-4 peptides has been shown to decrease peptide-mediated cAMP responses with even greater loss in $_iCa^{2+}$ signalling [175-177]. In addition to forming homodimers, the GLP-1R has been demonstrated to form heterodimers with other family B GPCRs, particularly with GIPR. The heterologous coexpression of the GLP-1R and GIPR leads to the changes in $_iCa^{2+}$ mobilization and GLP-1-

induced β-arrestin-1 recruitment [173]. These findings show that heterodimerization may play an important role in ligand-induced signalling bias because these two receptors are physiologically coexpressed in pancreatic β-cells.

1.12 GLP-1R Polymorphisms

Single-nucleotide polymorphisms (SNPs) occur in the genes encoding GPCRs and provide the potential to genetically modify receptor function in families or populations. While many of these polymorphisms are silent and do not change the wild-type receptor phenotype, it is well known that missense mutations in GPCRs can result in a variety of pharmacological abnormalities, including alterations in basal activity, ligand binding, receptor expression and downstream pathway signalling, potentially leading to disease or altered response to therapy in the affected individual [178]. Examples of this include the human calcium-sensing receptor (CaSR), a GPCR which senses extracellular levels of calcium ion and whose primary role is the regulation of extracellular Ca^{2+} by inhibition of parathyroid hormone (PTH) secretion. SNPs in CaSR have been shown to influence the development of hyper- (loss of function mutations) and hypocalcemia (gain of function mutations) [179]. These conditions are treatable with calcimimetics or calcilytics respectively [180]. Jensen's metaphyseal chondrodysplasia, a rare form of short-limbed dwarfism has been associated with the presence of at least 1 of 3 SNPs in the parathyroid hormone PTH1 receptor [161]. In a population study, an increase in late onset T2D incidence was observed in subjects possessing a SNP (G40S) in the glucagon receptor that significantly reduces glucagon binding affinity *in vitro* [181, 182]. Thus, it is becoming increasingly important to identify receptor variants that are associated with onset disease and effectiveness of drug treatments.

The recently published genetic variant database from the Exome Aggregation Consortium (ExAC) has revealed the existence of multiple SNPs in GLP-1R (**Figure 11**) [183], particularly in domains susceptible to mutation-induced pharmacological alteration. These include the receptor N-terminus, a well-established site of GLP-1 binding [184], and IC domains (loops 1, 2, and 3 and the C terminus) implicated in G-protein coupling [92]. To date, ten GLP-1R variants (**Table 1**) have been assessed *in vitro*, and explored in a wide range of functional outputs [170, 185-187].

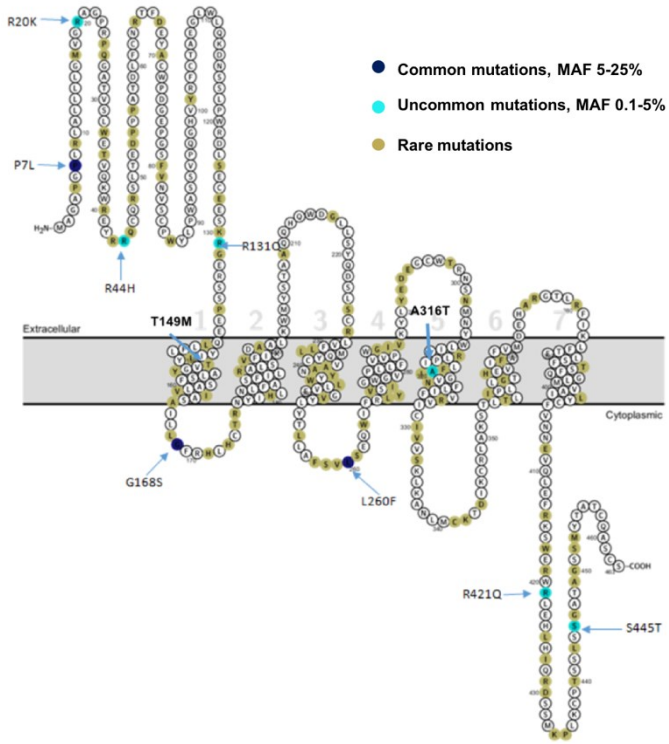

Figure 11. Amino acid sequence of hGLP-1R showing residues affected by SNPs. A structure plot of GLP-1R was created using the Protter software [81]. SNPs are reported in the Exome Aggregation Consortium (ExAC) database [188]. Two T2D-associated GLP-1R variants are highlighted in bold (T149M and A316T). (Data from [189], downloaded 12-01-2016)

Of these, one polymorphic variant (T149M) has been shown to significantly lower GLP-1 binding activity *in vitro* resulting in a 30-fold reduction in GLP-1R potency [77, 185]. It is now widely accepted that this variant leads to attenuated receptor function *in vitro* and has been reported to have an association with high risk of T2D.

Comparative analysis of the effect of different GLP-1R polymorphic variants on the cAMP, Ca^{2+} and pERK1/2 pathways revealed that all other mutants had no effect on GLP-1 action [187]. Although different ligands were shown to have similar trend in activation of cAMP and pERK1/2 for receptor variants, each ligand's efficacy in Ca^{2+} mobilization varied for each variant [187]. The effect on $_{i}Ca^{2+}$ signalling was firstly suggested to be dependent on the cell surface expression of the mutant receptors. However, additional studies, including GLP-1R dimerization studies [175] have shown that $_{i}Ca^{2+}$responses were much strongly attenuated than cAMP or ERK1/2 signalling while the receptor was forming the homodimers. This

finding suggested that coupling to $_{i}Ca^{2+}$ signalling is much more sensitive to conformational changes in the GLP-1R.

Residue Position in GLP-1R	Nucleotide substitution	Amino Acid substitution	Frequency of Occurrence		NCBI Identification Number	T2D risk association
			Homozygous	Heterozygous		
7	C**C**G→C**T**G	Pro→Leu	0.08	0.37	rs10305420	n/a
20	A**G**G→A**A**G	Arg→Lys	0.00011	0.0074	rs10305421	n/a
44	C**G**C→C**A**C	Arg→His	0.0001	0.0036	rs2295006	n/a
131	C**C**A→C**A**A	Arg →Gln	0.0035	0.0413	rs3765467	n/a
149	A**C**G→A**T**G	Thr→Met	0	1.503e-05	112198	High risk [77, 185]
168	**G**GC→**A**GC	Gly →Ser	0.034	0.2	rs6923761	n/a
260	TT**C/**TT**T**→TTA	Phe→Leu	0.159	0.405	rs1042044	n/a
316	**G**CC→**A**CC	Ala →Thr	0.00013	0.0103	rs10305492	Low risk [13, 14]
333	T**C**C→T**G**C	Ser→Cys	0	4.119e-05	rs10305493	n/a
421	C**G**G→C**A**G	A**rg**→Gln	1.6e-05	0.002	rs10305510	n/a

Table 1. Functionally characterised SNPs in the human *GLP-1R*. Allele frequency data are reported from [189].

Several studies have been performed to find the association of the GLP-1R variants with T2D and other metabolic diseases [13, 14]. To date, only two coding mutations have been found to be associated with T2D: 112198 (T149M) and rs10305492 (A316T) (**Table 1**). The variant rs10305492 that changes Ala^{316} to Thr was recently shown to alter T2D risk [13]. The authors combined data from 23 studies comprising up to 60,564 non-diabetic individuals of European and African ancestry and conducted gene-based analyses for fasting glucose (FG) and fasting insulin (FI). As a result, the rs10305492 minor allele has been associated with lower FG, and lower T2D risk. The FG-lowering allele was also associated with higher 2-h glucose levels and a lower insulinogenic index, indicating lower early insulin secretion. However, the mechanism which leads to the decreased diabetes risk was not elaborated in the paper.

A subsequent study showed that the rs10305492 minor allele was also associated with protection against coronary heart disease [14]. Since the mutant variant rs10305492 in *GLP-1R* mirrors the action of GLP-1R agonists in lowering fasting glucose level and T2D risk, the

authors suggested that this SNP could be used as a tool to predict cardiovascular safety of these agents. Ongoing clinical trials evaluating cardiovascular risk of GLP-1R agonists will provide proof of this concept and will also augment the evidence on the broader validity of genetic approaches in drug-target validation at an early stage in drug development.

1.13 BRET assays to study GPCRs signal transduction and signalling bias

Highly sensitive assays and techniques are required for studying GPCRs signal transduction and biased signalling mechanisms. One of the approach that is commonly used nowadays, is Bioluminescence Resonance Energy Transfer (BRET), a sensitive and non-destructive method to examine protein–protein interactions in live cells [190]. BRET is based on the transfer of energy between a donor (luciferases) and an acceptor (fluorescent proteins). Degradation of a luminescent substrate results in light emission which in turn excites the GFP leading to fluorescence emission.

In most BRET studies, the donors are variants of the *Renilla reniformis* luciferase enzyme (RLuc) with its substrate coelenterazine, and the light emitting acceptors are the variants of green fluorescent proteins (GFPs) [191]. Upon substrate addition, RLuc emits light, which in turn excites the GFP by resonance energy transfer if the luciferase and fluorophore are in close proximity (within a radius of approximately 50 Å). Different generations of BRET (BRET1, BRET2) have been developed, depending on the type of enzyme substrate and the nature of donor/acceptor pairs (**Figure 12**) [190, 192-196]. The original BRET method, using coelenterazine *h* (benzyl-coelenterazine) as a substrate, is called BRET1 [197]. In BRET1, the maximal emission of RLuc is observed at 480 nm, wavelength that is appropriate for excitation of a yellow fluorescent protein (enhanced YFP, eYFP), which subsequently reemits light at 530 nm (**Figure 12**). BRET1 is characterized by strong signal and long life-time, and it is mostly used for BRET saturation assays [198]. Changes in the RLuc substrate have led to the second generation of BRET, the BRET2. In this method, the substrates bisdeoxycoelenterazine (DeepBlueC) or didehydrocoelenterazine (coelenterazine-400a) are used to replace coelenterazine *h.* The emission spectra of RLuc shifts to 400 nm with the appropriate acceptors GFP2 and GFP10 characterized by the emission spectra of 510 nm (**Figure 12**). Compared with BRET1, BRET2 has an improved separation of donor and acceptor emission peaks making this method a better choice for screening assays where high signal to noise ratios are required [198]. When GFP10 is located within a distance of up to

~100 Å, it is excited and emits light at a wavelength of 510 nm. The BRET2 signal is then measured as the ratio 400 nm/510 nm [194]. The BRET2-based approach is much more sensitive and is optimal for detection of weak BRET signals. Several studies continue to improve the potential use of other luciferases and new coelenterazine derivatives with brighter or extended light emission resulting in introducing BRET3, eBRET2 (enhanced BRET2), eBRET (extended BRET1), and QD-BRET (Quantum Dot-BRET) [198].

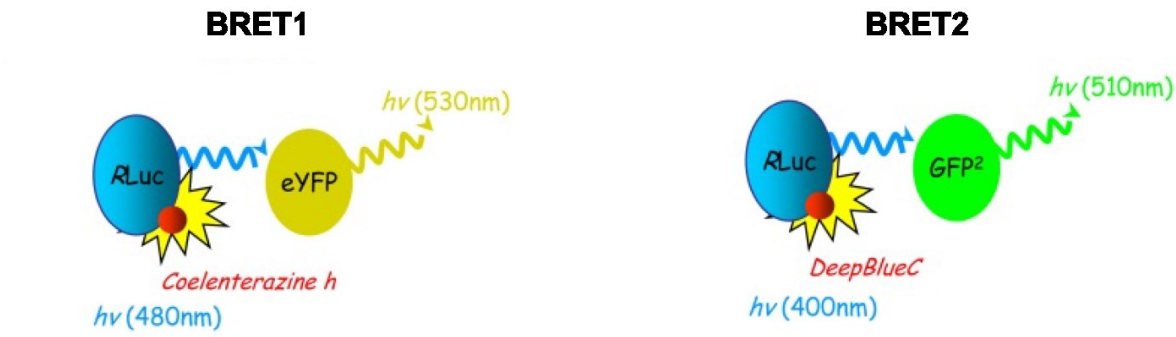

Figure 12. The basics of BRET1 and BRET2. BRET1 and BRET2 are based on the use of different coelenterazine substrates (Coelenterazine *h* for BRET1 and DeepBlueC in BRET2). These substrates confer specific spectral properties to *Renilla* Luciferase and the energy acceptor then is chosen correspondingly to the emission wavelength of *Renilla* Luciferase (eYFP in BRET1 and GFP10 or GFP2 in BRET2). Adapted from [199].

The BRET response depends on the distance and the relative orientation of the donor and acceptor pair. Usually, two proteins that potentially participate in signalling complexes are fused separately to a donor and an acceptor. Sometimes, when subtle changes in protein conformation upon complex assembly or disassembly are studied, proteins are modified by labeling with both BRET donor and acceptor moieties engineered into the same protein [191, 200].

A major advantage of the BRET technique over other methods is that it does not require any external illumination to initiate the energy transfer, which reduces background noise that results from direct excitation of the acceptor or from photobleaching [197]. In addition, BRET experiments can be conducted under conditions that more closely reflect the biochemical environments occurring in living organisms. Because of its fast reaction kinetics, BRET allows for real time detection of complexes or conformational changes that may be

transient [201]. As a result, BRET has become a widely used experimental technique of choice to identify and monitor dynamics of protein-protein interactions between GPCRs, G proteins, and β-arrestins; as well as to measure formation and accumulation of second messengers following the receptor activation [191]. More recently, several studies have applied BRET for the study of dynamic cellular processes, such as the modulation of the interaction of two proteins following a pharmacological treatment or the development of biosensors for various signalling pathways [191].

The first and most reported BRET-based sensor studies for GPCRs have been investigations into the dynamics of β-arrestin recruitment [191]. In 1997, Barak et al. showed for the first time that stimulation of β2 adrenergic receptor (β2AR) with the full agonist isoproterenol resulted in the recruitment of GFP-tagged β-arrestin 2 to the plasma membrane [202]. Over years of extensive studies, it has become clear that G protein-and β-arrestin-dependent signalling pathways may lead to different physiological effects. This resulted in the idea that the selective modulation of either the G protein or the β-arrestin-dependent pathway can be used as therapeutic approaches [203]; and new techniques for investigation of β-arrestin signalling as an important avenue for drug development have been subsequently developed. Considering the many advantages of BRET, the measurement of β-arrestin recruitment to receptors using BRET assays became one of the main approaches. The first study investigating β-arrestin recruitment to a GPCR using BRET was performed with β-arrestin 2-YFP and β2AR–Rluc pair [191]. The versatility of BRET assay based on β-arrestin 2 recruitment approach was also successfully demonstrated in a high-throughput screening (HTS) platform to identify new antagonists of the chemokine CCR5 receptor [204].

Recently, BRET-based biosensors were used to assess signalling bias between different Angiotensin II type 1 receptor (AT1R) ligands [201]. The functional selectivity between $G\alpha_q$ and β-arrestin 2-dependent responses were quantified using the operational model described by Kenakin et al. [155]. According to this method, the full concentration-responses of different ligands measured for distinct signalling pathways are compared with that of a reference ligand, usually endogenous ligand. This helps to evaluate whether unknown ligands have distinct biases toward one pathway opening a promising avenue for improving drug efficacy and therapy [201].

In the following chapters of this thesis, we will employ BRET biosensors to evaluate how the T2D-risk lowering rs10305492 (A316T) alters signalling by GLP-1R in cultured cell lines.

GLP-1R is a major therapeutic target for the treatment of T2D and obesity and is also being actively pursued for novel therapies in the treatment of various neurodegenerative and cardiovascular diseases. The surprising beneficial action of the rs10305492 (A316T) variant in reducing T2D risk justifies our detailed study of how this variant changes the activity of GLP-1R signalling pathways. Since GLP-1R signalling involves several different physiological ligands and downstream pathways, our approach is designed to highlight the potential importance of differential modulation of these pathways by the GLP-1R (A316T) mutant receptor in exerting its effects.

CHAPTER 2. Assessing the Changes in GLP-1R(A316T) Signalling

2.1 Materials and Methods

Materials

GLP-1R ligands, exendin-4 (Exe-4) and glucagon were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Dulbecco's modified Eagles medium (DMEM), fetal bovine serum (FBS) and other cell culture reagents were purchased from ThermoFisher Scientific (Waltham, MA, USA).

Plasmids and Constructions

The full-length human GLP-1R cDNA was amplified from the Mammalian Gene Collection library clone BC112126 (BCCA Genome Science Centre, Vancouver, Canada) by polymerase chain reaction (PCR). C-terminal GFP10 or RLucII fusions of GLP-1R (GLP-1R-GFP10 and GLP-1R-RLucII), SP-FLAG-GLP-1R, and all GLP-1R mutant constructs were generated using standard molecular biology methods. The coding sequences were subcloned into the multicloning site of the pcDNA5/FRT/TO vector under the control of a tetracycline-regulated promoter (CMV/TetO2). All cDNA clones were verified by sequencing and restriction digest and sequencing analysis were used to confirm the coding sequence of all expression vectors. Plasmids encoding GFP10-Epac-RLucII for cAMP-measurement as well as β-arrestin2-RLucII were obtained from Dr. Y. Namkung (Prof. S. Laporte's laboratory, Montreal, Canada). Plasmids encoding the fusion proteins rGFP-CAAX, rGFP-FYVE and PKC biosensors were also developed in Prof. S. Laporte's laboratory [201].

Cell culture and transient transfection

We used the Flp-In™ T-REx 293 cell line, which is designed for rapid generation of stable cell lines, to perform our GPCR functional assays. Flp-In T-REx 293 cells were routinely maintained and passaged in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin. Cells were grown at 37ºC in 5% CO_2 and 90%

humidity. For transient transfection experiments, cells were used at ~40% confluency and transfected using with TransIT-LT1 Transfection Reagent (Mirus Bio LLC, Madison, WI, USA) according to the manufacturer's protocol. The cells were imaged two days after transfection.

Human insulin-secreting 1.1B4 cells were maintained in RPMI-1640 tissue culture medium (37 ºC, 5% CO_2) containing 11.1 mmol/l glucose and 0.3 g/l of L-glutamine supplemented with 10% FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Maintenance at 11.1 mmol/l glucose in culture is optimal for function of these cells [205]. Cells at 60% of confluency were transfected using TransIT-LT1 Transfection Reagent (Mirus Bio LLC). Cells were processed or imaged 1-2 days after transfection.

For the BRET assays, cloned receptors were transiently expressed in either Flp-In T-REx 293 cells or 1.1B4 cells transfected using the calcium phosphate method. Cells were seeded at a density of 5 x 10^5 per 10-cm dish one day before transfection, and transfection was carried out according to published protocols [206]. The culture medium was replaced 18 h following transfection, and cells were divided for subsequent experiments. All assays were performed 48 h after transfection.

Stable cell lines

Flp-In T-REx 293 cells were transfected with wild type GLP-1R or A316T mutant GLP-1R expression vector and pOG44 using TransIT-LT1 according to the manufacturer's instructions (Mirus Bio LLC). After 48 h, the cells were grown in DMEM supplemented with 100 µg/ml Hygromycin B and 10 µg/ml Blasticidin (Invitrogen) to select cells in which recombination had occurred. Expression of GLP-1R was induced by the addition of 25 µg/ml tetracycline to the cell medium.

Imaging experiments

Stable T-REx 293 lines expressing an inducible wild type GLP-1R-GFP10 or mutant GLP-1R (A316T)-GFP10 were plated in 35 mm Fluorodishes with glass coverslips (World Precision Instruments, Sarasota, FL, USA) at a density of 100,000 cells per dish. The next day, tetracycline was added to induce the expression of GLP-1Rs. After next twenty-four hours,

the cells were analyzed on a DeltaVision microscope (GE Healthcare, formerly Applied Precision), and images were collected using a 60x oil immersion lens. For receptor internalization studies, exendin-4 (100 nM final concentration) was added to the dishes between 0 and 30 minutes prior to imaging.

Live-cell surface flow cytometry (FACS)

Flp-In™ T-REx 293 cells were transfected with SP-FLAG-GLP-1Rwt and SP-FLAG-GLP-1(A316T) using TransIT-LT1 Transfection Reagent (Mirus Bio LLC). One day after transfection, the cells were washed with cold PBS and detached using PBS-EDTA. The cell suspension in PBS-2% BSA was incubated at room temperature for 1 h with primary antibody (1:250 mouse anti-FLAG M2, Sigma). After two washings with PBS, the cells were resuspended in the secondary antibody solution PBS-BSA (1:1000 goat polyclonal anti-mouse Alexa Fluor® 488, Invitrogen) and incubated at room temperature for 45 min in dark. After each step, a control sample was collected and used to determine the desired ranges and parameters in flow cytometry analysis. The cell pellet was washed twice with PBS and transferred into FACS tubes. Cell analysis was performed using BD FACSCANTO II cytometer (BD Bioscience).

BRET2 measurements

Flp-In T-REx 293 cells were transfected with GLP-1R (1 μg), a BRET donor (90 ng of β-arrestin2-RLucII) along with 480 ng of BRET acceptor (rGFP-CAAX or rGFP-FYVE). For EPAC experiments GLP-1R and GFP10-EPAC-RlucII were used at 2:1 ratio. A vector without an insert was used to ensure a constant amount of total DNA in all parallel transfections. Flp-In T-REx 293 cells stably expressing wild-type or mutant GLP-1R were transfected with BRET sensors using the same conditions. One day following transfection, the cells were detached and seeded onto poly-L-ornithine coated 96-well white plates at a density of 35,000 cells per well. Human insulin-secreting 1.1B4 cells were seeded before transfection onto poly-ornithine-coated 96-well white plates at a density of 30,000 cells per well. After 24 h, cells were co-transfected with GLP-1R and biosensors using TransIT-LT1 Transfection Reagent according to the manufacturer's instructions (Mirus Bio LLC).

One day following transfection, the cells were washed once with pre-warmed Tyrode's buffer (140 mM NaCl, 2.7 mM KCl, 1 mM $CaCl_2$, 12 mM $NaHCO_3$, 5.6 mM D-glucose, 0.5 mM $MgCl_2$, 0.37 mM NaH_2PO_4, 25 mM HEPES at pH 7.4), and then stimulated with various concentrations of ligands in Tyrode's buffer. Coelenterazine 400a at 5 μM final concentration was added 2-4 minutes before BRET measurements. For the EPAC, β-arrestin2 and PKC assays, BRET signals were measured after adding Coelenterazine 400a, followed by various concentrations of Exe-4 for 10 minutes. For the endocytosis experiments using rGFP-FYVE and GLP-1-RLucII, cells were incubated for 1 h after addition of various concentrations of Exe-4. Coelenterazine 400a was added 3 minutes before measuring BRET.

All the BRET measurements were performed using a Synergy2 (Biotek®) microplate reader with a filter set (centre wavelength/band width) of 410/80 nm (donor) and 515/30 nm (acceptor), for detecting the RLucII *Renilla* luciferase (donor) and GFP10 or rGFP (acceptor) light emission. All measurements were made at room temperature. The raw BRET ratio was determined by calculating the ratio of the light intensity emitted by GFP10 or rGFP divided by the light intensity emitted by RLucII. Internalization-promoted BRET signals were normalized to basal BRET ratios (without ligand) and plotted as a per cent basal using the formula $(BRET_{ligand}/BRET_{basal})x100$, so that basal BRET is set at 100%.

Data Analysis

The acceptor/donor ratio was plotted as a function of ligand concentration and data was analyzed using nonlinear regression by GraphPad Prism5 software (GraphPad Software, Inc., San Diego, CA, USA). Estimation of the EC_{50} values for ligand-mediated β-arrestin2 recruitment were calculated using this GraphPad Prism software. The dose-response curves represented throughout this study represent the best fits generated using the GraphPad software.

2.2 Results

2.2.1 Evaluation of cell surface expression of the human GLP-1R (A316T) variant

We first studied GLP-1R expression and trafficking in the Flp-In™ T-REx 293 cell line, which contains an inducible expression system and a FRT docking site for the Flp recombinase. In this system, the gene of interest is incorporated into a specific site of the genome under the control of a tetracycline-regulated promoter. The T-REx 293 docking site is flanked by a tetracycline repressor element (tetR), which inhibits transcription from the transgene. Addition of tetracycline inactivates the repressor and leads to the expression of targeted protein at tightly controlled levels. To obtain a stable line with inducible GLP-1R expression, cDNA encoding GLP-1R-GFP10 wild type and GLP-1R-GFP10 (A316T) were cloned into pcDNA5/FRT/TO vector under the control of a tetracycline regulated promoter. Flp-In™ T-REx 293 cells were transfected and antibiotic selection was performed. Expression of GLP-1R was activated in the presence of tetracycline and imaged twenty-four hours after induction. Live cell imaging studies showed that the Ala316Thr mutation position results in a reduction of a receptor expression at the cell surface (**Figure 13**).

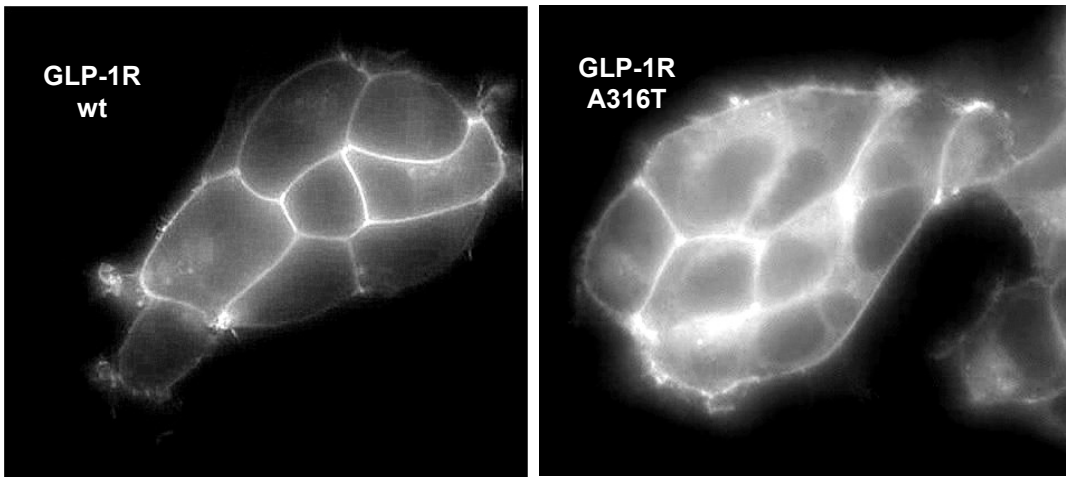

Figure 13. GLP-1R wild type and GLP1R (A316T) cell surface expression profile in Flp-In™ T-REx 293 cells. Flp-In T-Rex 293 cells stably expressing GLP-1R variants were plated on glass cover slips and incubated overnight following treatment with tetracycline. Twenty-four hours after induction of GLP-1R expression cells were mounted on the stage of DeltaVision microscope (60x magnification). Images were analyzed with ImageJ software. Images are representative of the three independent experiments (Scale bars = 15µm).

To further evaluate the level of cell surface expression profile for the mutant GLP-1R (A316T) we used fluorescence-activated cell sorting (FACS), a sensitive, quantitative and convenient method to quantify the levels of GPCR cell surface expression. Taking into the account the fact that the mature GLP-1R is expressed at the cell membrane without the signal peptide (SP) (1-23 aa) [93, 207], we designed new constructs containing FLAG sequence at the N-terminus downstream from the SP sequence (SP-FLAG-GLP-1Rwt and SP-FLAG-GLP-1R (A316T)). We expected that after the signal peptide has been cleaved, the N-terminally FLAG tagged receptor would traffic to the cell membrane allowing the receptor surface expression to be evaluated by FACS. HEK293 cells transfected with these constructs were analyzed for GLP-1R cell surface expression by flow cytometry, using the anti-FLAG antibodies, acquiring the non-stained cells control first, then the secondary antibody control and the 293T-stained cells, all in triplicate. Representative example of FACS histogram generated by staining HEK 293T cells using the mouse anti-FLAG and anti-mouse Alexa Fluor® 488 antibodies are shown in **Figure 14A**.

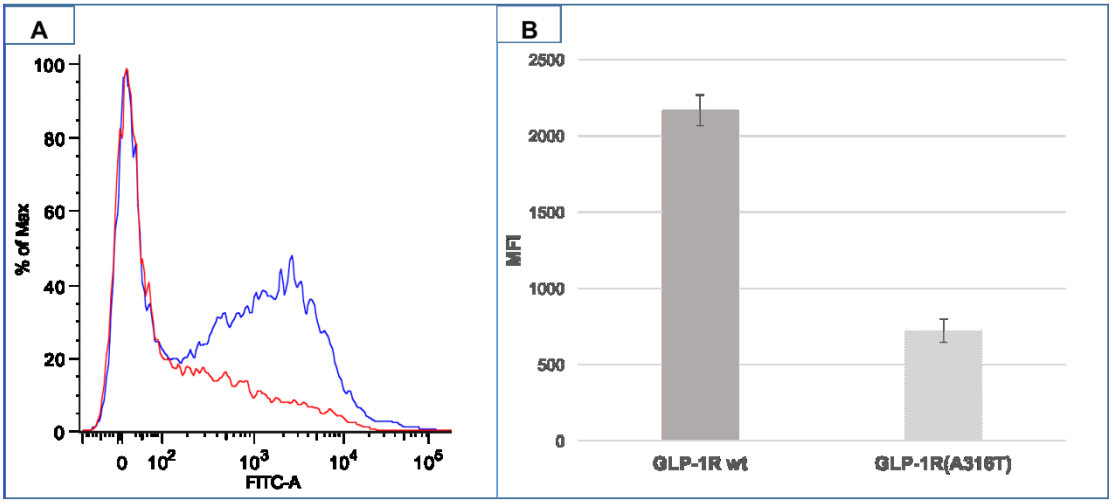

Figure 14. Detection of GLP-1R on the surface of 293T cells using FACS staining. (**A**) Cell surface receptor levels measured by FACS for cells expressing GLP-1R wt (blue) and the GLP-1R (A316T) mutant (red). (**B**) Summary of the corresponding mean fluorescence intensities (MFI). Figure is representative of 3 independent experiments.

As expected, the absolute mean fluorescence intensity observed in GLP-1R (A316T)-expressing cells was three times lower of that observed for the cells expressing wild-type

GLP-1R (**Figure 14B**). This data confirms that the mutant receptor is expressed at the membrane of 293T cells at 30% of wild type levels.

As GLP-1R is expressed in pancreatic β-cells, it was also important to examine how hGLP-1R wt and GLP-1R (A316T) are expressed on the cell membrane of the pancreatic cell line. We choose the human insulin-secreting 1.1B4 cell line, which were generated by electrofusion of normal human islet cells with human PANC-1 pancreatic adenocarcinoma cells [208]. This cell line exhibits many of the functional attributes of pancreatic β-cells, including insulin secretion. When expressed in 1.1B4 cells, the GLP-1R (A316T) mutant showed significantly less cell surface expression than the wild type receptor and was predominantly located intracellularly (**Figure 15**).

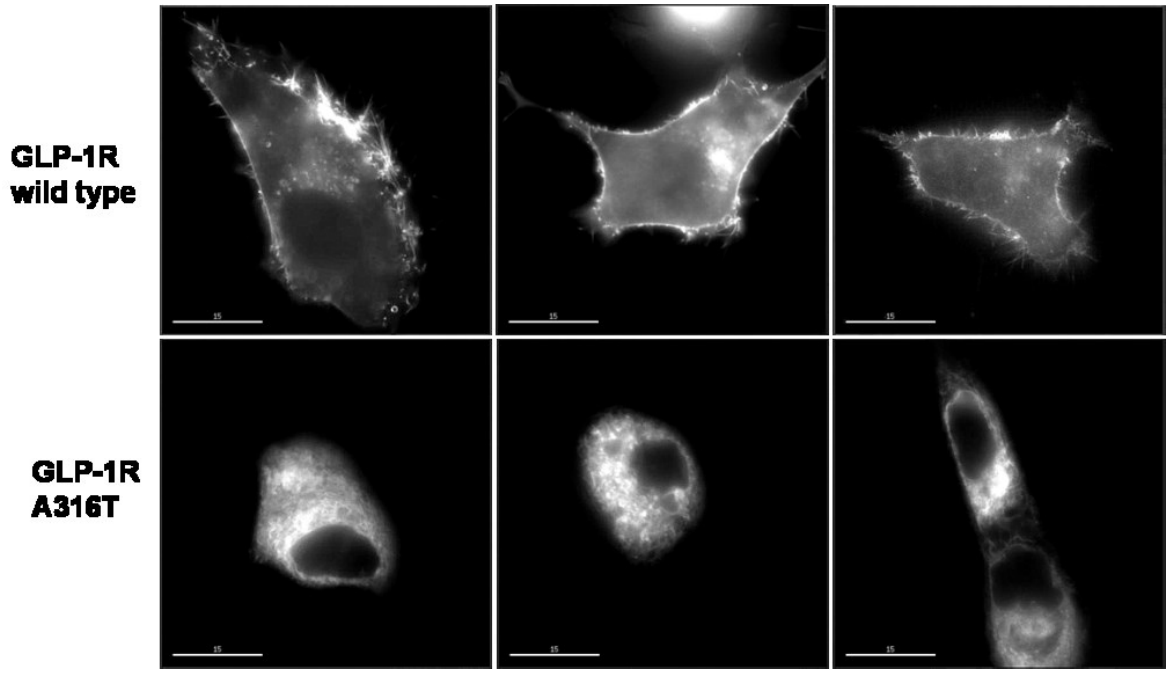

Figure 15. hGLP-1R cell surface expression profile in 1.1B4 pancreatic cells transiently expressing wild type and mutant GLP-1R. 1.1B4 cells were plated on the dishes with glass cover slips and transiently transfected with GLP-1R-GFP10 wt or GLP-1R (A316T)-GFP10. Twenty-four hours after transfection, the live cells were imaged using a DeltaVision microscope (60x magnification). Images are representative of the three independent experiments (Scale bars = 15µm).

2.2.2 Assessing the changes in GLP-1R(A316T) downstream signalling pathway

To assess potential changes in GLP-1R function caused by the A316T mutation, the mutant receptor was further characterized *in vitro* using specific live-cell assays that monitor receptor signaling. Cell-based assays for $G\alpha_s$-protein binding (cAMP production), $G\alpha_q$-protein binding (intracellular Ca^{2+} mobilization) and β-arrestin recruitment were used to determine the activity of the receptor relative to the wild type. To monitor these main pathways in living cells, we quantitatively assessed GLP-1R signalling using a BRET2 technique.

a. The GLP-1R A316T mutant shows normal cAMP accumulation

cAMP is a principal signalling molecule generated by the ligand binding and activation of class B GPCRs. Therefore, intracellular cAMP accumulation after stimulation of adenylate cyclase following $G\alpha_s$ activation is the best characterized GLP-1R signalling event. In our experiments, we used an EPAC-based biosensor described previously [209], which contains a mutant form of human EPAC1 inserted between GFP10 and RLucII. In the absence of cAMP, the RLucII and GFP10 regions of the biosensor are in a close proximity and BRET2 can be measured upon addition of coelenterazine 400a (**Figure 16**). cAMP binding to GFP10-EPAC-RLucII induces a conformational change in EPAC resulting in a decrease in the BRET2 signal between donor (RLucII) and the acceptor (GFP10).

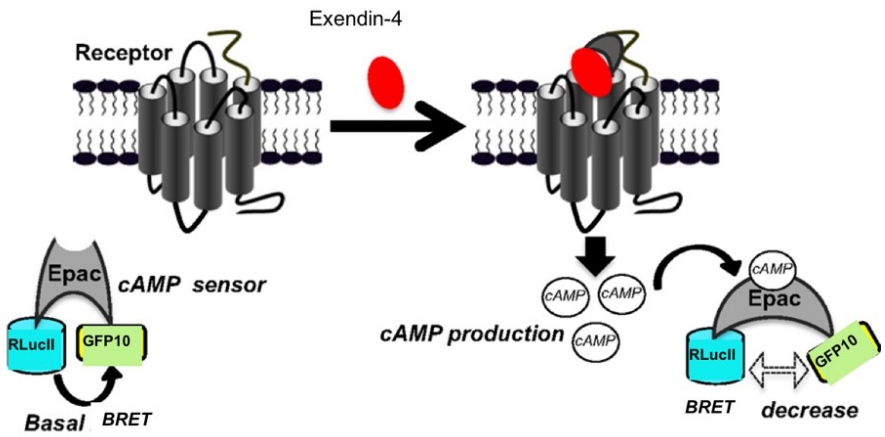

Figure 16. BRET EPAC biosensor for cAMP monitoring. EPAC, a protein that changes conformation upon cAMP binding, was tagged with a BRET donor (RLucII) and a BRET acceptor (GFP10) on each extremity of the protein to monitor intramolecular BRET signal.

Our experiments showed that stimulation of HEK293 cells coexpressing the EPAC biosensor and both forms of GLP-1R with different concentrations of GLP-1R ligand, Exe-4, lead to a concentration-dependent cAMP response (**Figure 17**). Surprisingly, the A 316 T mutant receptor did not show any suppression of cAMP accumulation compared to the wild type receptor. The cAMP levels induced by Exe-4 stimulation were similar for both types of receptor, indicating that the A316T mutation of the receptor had no effect on $G\alpha_s$-activation in both HEK293 cells despite its decreased cell surface expression (**Figure 17A**) and 1.1B4 cells (**Figure 17B**). This suggests that another GPCR-activated pathway most likely explains the effect of the A316T mutation in lowering T2D risk.

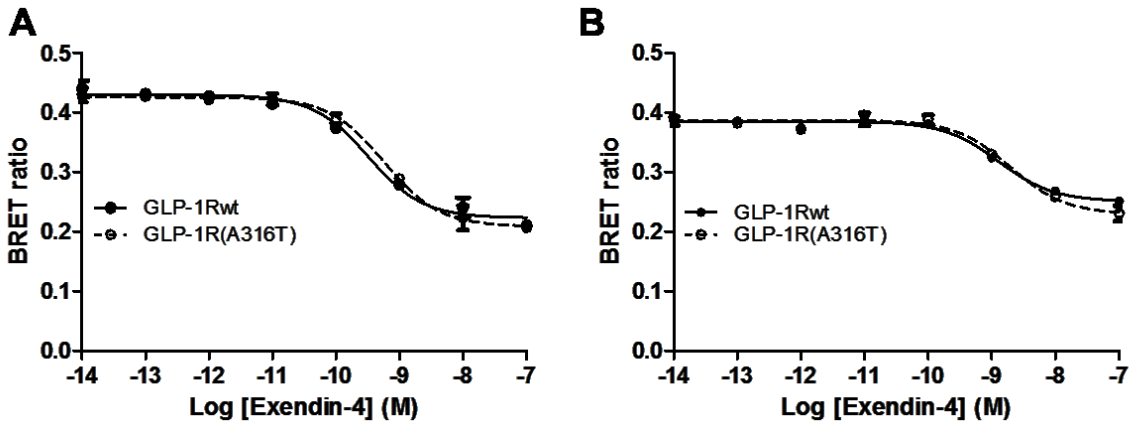

Figure 17. Concentration-response curves for Exe-4 induced increase in cAMP production. Measurements were performed after 7 min of stimulation in Flp-In T-REx 293 cells stably expressing the human GLP-1Rwt or mutant GLP-1R (A316T) and transiently transfected with GFP10-EPAC-RLucII (**A**) or in insulin-secreting human 1.1B4 cells transiently transfected with GLP-1Rwt or GLP-1R (A316T) and GFP10-EPAC-RLucII (**B**). The data are mean ± SEM of a typical experiment that was performed independently at least three times.

b. The GLP-1R A316T mutation leads to decreased intracellular Ca^{2+} mobilization

We next investigated the ability of mutant GLP-1R (A316T) to promote GLP-1R mediated intracellular Ca^{2+} mobilization. It is generally accepted that the GLP-1R-mediated increase in $[Ca^{2+}]_i$ in β-cells is caused by GLP-1R activation *via* the $G\alpha_s$ pathway through cAMP- and PLC/Ca^{2+} dependent mechanisms. However, an increasing number of studies have shown

that increased levels of $[Ca^{2+}]_i$ can be inhibited by the selective $G\alpha_q$ inhibitor, confirming that GLP-1R can also functionally couple to $G\alpha_q$ [124, 210].

$G\alpha_q$ signalling activates phospholipase C (PLC), which catalyzes the cleavage of membrane-bound PIP_2 into the second messengers IP_3 and DAG. Binding of IP_3 to its receptor on the ER results in calcium release; and increased concentrations of calcium and DAG lead to the activation of PKC. Consequently, BRET biosensors monitoring PKC activity have been developed in the Laporte laboratory in order to measure calcium flux following GPCR activation. HEK293 cells overexpressing the mutant GLP-1R (A316T) showed decreased PKC activity upon receptor stimulation when compared with the wild type receptor, reflecting decreased intracellular Ca^{2+} levels (**Figure 18**). Previously published data by Koole et al. suggests that $_iCa^{2+}$ signalling depends not only on the cell surface expression of the receptor variant but also on the conformational changes in GLP-1R. This is also evident from studies on dimerization of the GLP-1R, in which Ca^{2+} responses were much more attenuated than other GLP-1R pathways [177]. Considering this, we suggested that A316T mutation results in a receptor conformation that is not favorable to the $G\alpha_q$ binding.

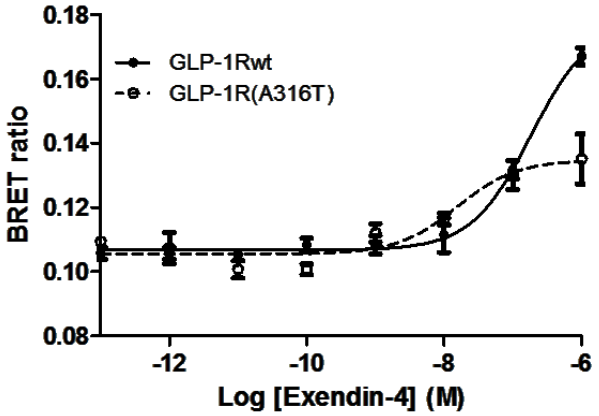

Figure 18. Concentration-response curve for Exe-4-induced PKC activation in Flp-In T-REx 293 cells stably expressing the human GLP-1Rwt or GLP-1R (A316T) and transiently transfected with GFP10-PKC-RLucII. Data from one representative experiment are shown (n=3). The data are mean ± SEM of a typical experiment that was performed independently at least three times.

c. The GLP-1R A316T mutation leads to greatly reduced β-arrestin 2 recruitment

Previous reports have demonstrated that GLP-1R directly couples to β-arrestin 1 and 2 following activation with Exe-4 [7, 211]. Therefore, we examined the ability of the mutant GLP-1R (A316T) to recruit β-arrestin using BRET2 assay with two biosensors, 1) RLucII-β-arrestin 2; and 2) a mutant version of the CAAX protein anchored to the internal face of the cell surface membrane and fused with *Renilla reniformis* green fluorescent protein rGFP [201]. When RLucII-β-arrestin 2 comes close to the cell membrane following GLP-1R activation, rGFP on cell membrane emits light resulting in an increase in BRET ratio. The mutant GLP-1R (A316T) showed a greatly diminished efficacy and potency for β-arrestin 2 recruitment, when compared with the wild type receptor, supporting our hypothesis about the changes in the mutant receptor conformation in both HEK293 (**Figure 19A**) and 1.B4 cells (**Figure 19B**).

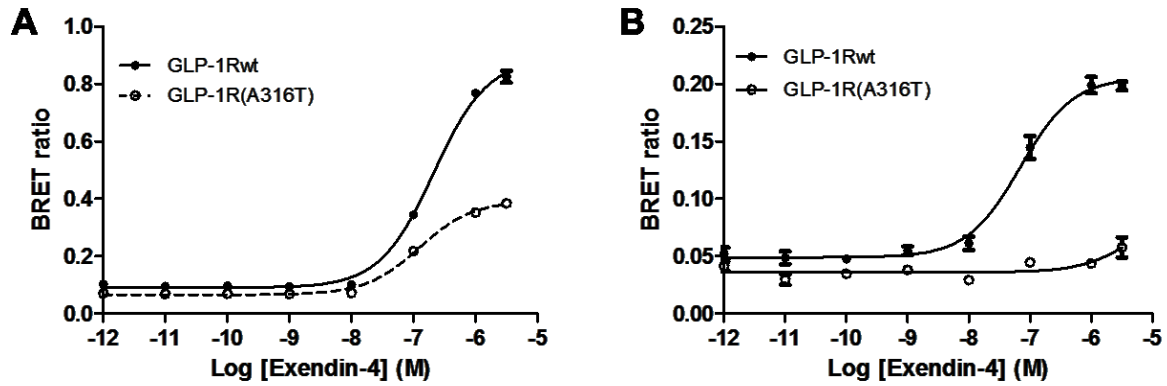

Figure 19. Concentration-response curve for Exe-4-induced β-arrestin 2 recruitment by GLP-1Rwt or mutant GLP-1R (A316T). (A) Flp-In T-REx 293 cells stably expressing the human GLP-1Rwt or mutant GLP-1R (A316T) were transiently transfected with RLucII-β-arrestin 2 and GFP10-CAAX.**(B)** Insulin-secreting human 1.1B4 cells were transiently transfected with GLP-1Rwt or mutant GLP-1R (A316T) and RLucII-β-arrestin 2 and GFP10-CAAX. The data are mean ± SEM of a typical experiment that was performed independently at least three times.

d. Coexpression of GRK2 does not increase β-arrestin2 recruitment by GLP-1R A316T

GPCR kinase (GRK)-mediated receptor phosphorylation is known to precede β-arrestin recruitment to the activated GLP-1R. A recent study of the β-arrestin 2-GLP-1R interaction

has shown that co-expression of GRK2 with β-arrestin 2, enhances β-arrestin 2 recruitment to the receptor [212]. In addition, the ability of GRK2 to attenuate GLP-1R-mediated cAMP accumulation in result of $G\alpha_s$ uncoupling has been demonstrated previously [126]. These results have confirmed the role of GRK2 as a promoter of β-arrestin 2 recruitment and subsequent G protein uncoupling from GPCR. Additionally, β-arrestin 2 interaction with GLP-1R has been shown to induce a special conformation of the receptor transmembrane domain which influences the N-terminal loop of the receptor responsible for the agonist binding [126]. Interestingly, this new conformation was shown to have increased affinity for glucagon, endogenous ligand for the glucagon receptor which acts as a full agonist of GLP-1R although with a very low affinity and potency.

We were interested to study whether GRK2 has the same effect on the mutant receptor. Our previous experiment (**Figure 19**) was performed in the presence of endogenous GRK2 levels which can be limiting in the case of GLP-1R overexpression. Thus, to compare β-arrestin interaction with both types of GLP-1R under exogenous GRK2 expression, we measured agonist-induced recruitment of RLucII-β-arrestin 2 to GLP-1R after co-expression of GRK2 (**Figure 20**). To activate the receptor, we used two agonists, exendin-4 and glucagon. Consistent with previously published results, GRK2 overexpression increased exe-4-induced

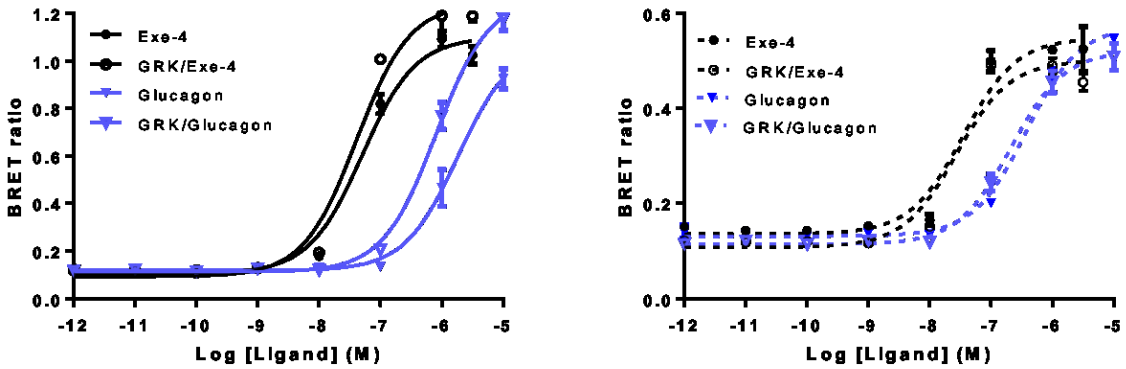

Figure 20. Concentration-response curve for β-arrestin 2 recruitment after activation with Exe-4 and glucagon. Flp-In T-REx 293 cells were transiently transfected with GLP-1R, RLucII-β-arrestin 2, rGFP-CAAX, with or without GRK. Results are shown for the wild type GLP-1R **(A)** and mutant GLP-1R (A316T) **(B)**. The data are mean ± SEM of a typical experiment that was performed independently at least three times.

β-arrestin recruitment to wild type GLP-1R compared with receptor without GRK2 coexpression (**Figure 20A, Table 2**). Glucagon is known to be a low-potency agonist on the wild type GLP-1R. However, upon coexpression with GRK2, a significant increase in potency of glucagon was observed. This confirmed the data by Jorgensen et al. [126] that GRK potentiates the β-arrestin recruitment to the wild type GLP-1R, which in turn leads to the stabilization of the receptor conformation resulting in higher affinity toward glucagon and unaltered affinity toward exe-4. Surprisingly, we observed that the activity of the mutant receptor was not altered by GRK2 overexpression (**Figure 20B**).

	EC_{50}, nm			
	Exendin		**Glucagon**	
	No GRK2	**GRK2**	**No GRK2**	**GRK2**
GLP-1R wt	50.33±8.7	40.89±10.5	1810±520	803.4±14
GLP-1R (A316T)	34.95±3.5	27.37±2.3	379.5±54.2	211±32

Table 2. Exendin-4 and glucagon induced β-arrestin 2 recruitment in 293 cells. All values are mean ± S.E.M. of 3 independent experiments.

e. The GLP-1R A316T mutation does not affect Exe-4-induced receptor internalization

As β-arrestin recruitment is believed to play a key role in GPCRs internalization, we further investigated the ability of the mutant GLP-1R (A316T) to internalize after ligand binding. The time dependent effect of Exe-4 on mutant hGLP-1R (A316T) internalization was determined by using live cell imaging. We treated Flp-In T-REx 293 cells stably expressing GLP-1R-GFP10 and GLP-1R (A316T)-GFP10 with ligand (10 nM Exe-4); and observed the internalization of cell surface GFP-tagged receptor (resulting in the appearance of intracellular punctate structures) using live cell fluorescence imaging. Our experiments showed that exendin-4 potently induced GLP-1R internalization in HEK293 cells. Unexpectedly, the mutant GLP-1R (A316T) appeared to be able to internalize as quickly as the wild-type GLP-1R (**Figure 21**).

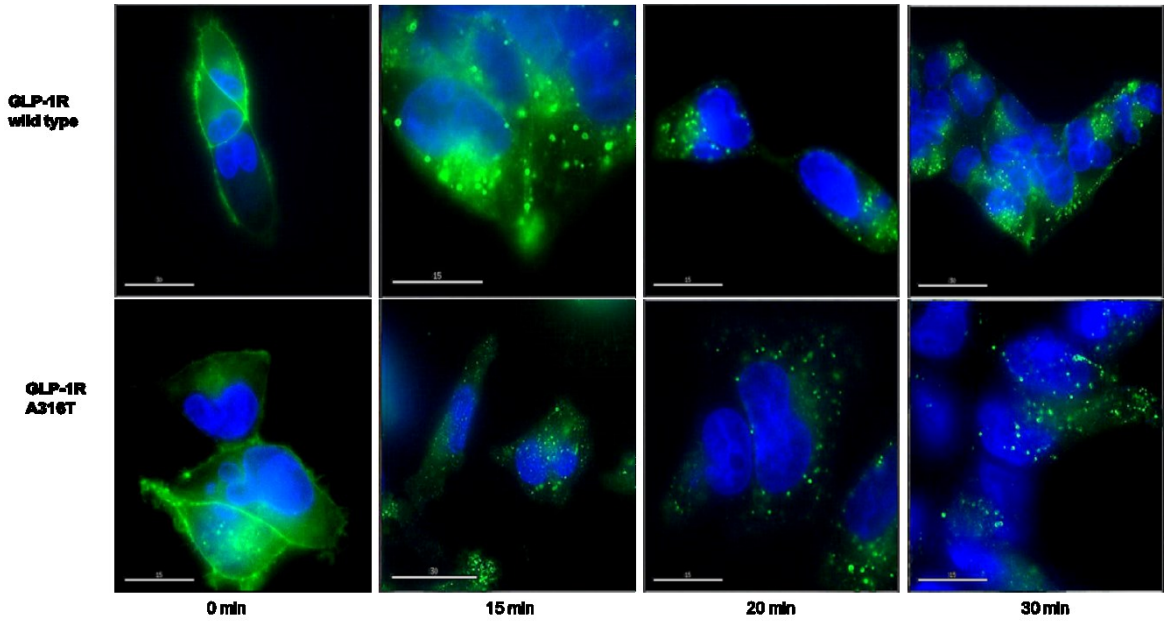

Figure 21. Time-dependent stimulation of hGLP-1R internalization by exendin-4 in HEK293 cells. HEK293 cells stably expressing GLP-1R variants were plated on glass cover slips and incubated overnight following treatment with tetracycline. DAPI staining (blue) was used to identify nuclei. To stimulate GLP-1R, 10 nM Exe-4 was applied. The cellular localization of fluorescently tagged receptors is shown before agonist addition (0 min) and 15, 20 and 30 min following agonist stimulation. GLP-1R internalization was visualized using a DeltaVision microscope (60x magnification) and analyzed with ImageJ software. Images are representative of the three independent experiments (Scale bars = 15µm)

The cellular internalization pattern of GLP-1R (wt or A316T) was further quantified by BRET2 assays using rGFP-CAAX as a plasma membrane marker and rGFP-FYVE as an early endosomal marker (both biosensors developed by Dr. Y. Namkung from the Laporte laboratory [201]). After stimulation with exendin-4, the receptor leaves the cell membrane increasing the distance between receptor and the marker rGFP-CAAX resulting in the decrease of the BRET signal for both wild type GLP-1R-RLucII and GLP-1R(A316T)-RLucII in HEK293 (**Figure 22A**) and 1.1B4 cells (**Figure 22B**). At the same time, the distance between the receptor and the early endosomal marker rGFP-FYVE decreases, leading to a larger BRET signal in HEK293 cells (**Figure 22C**).

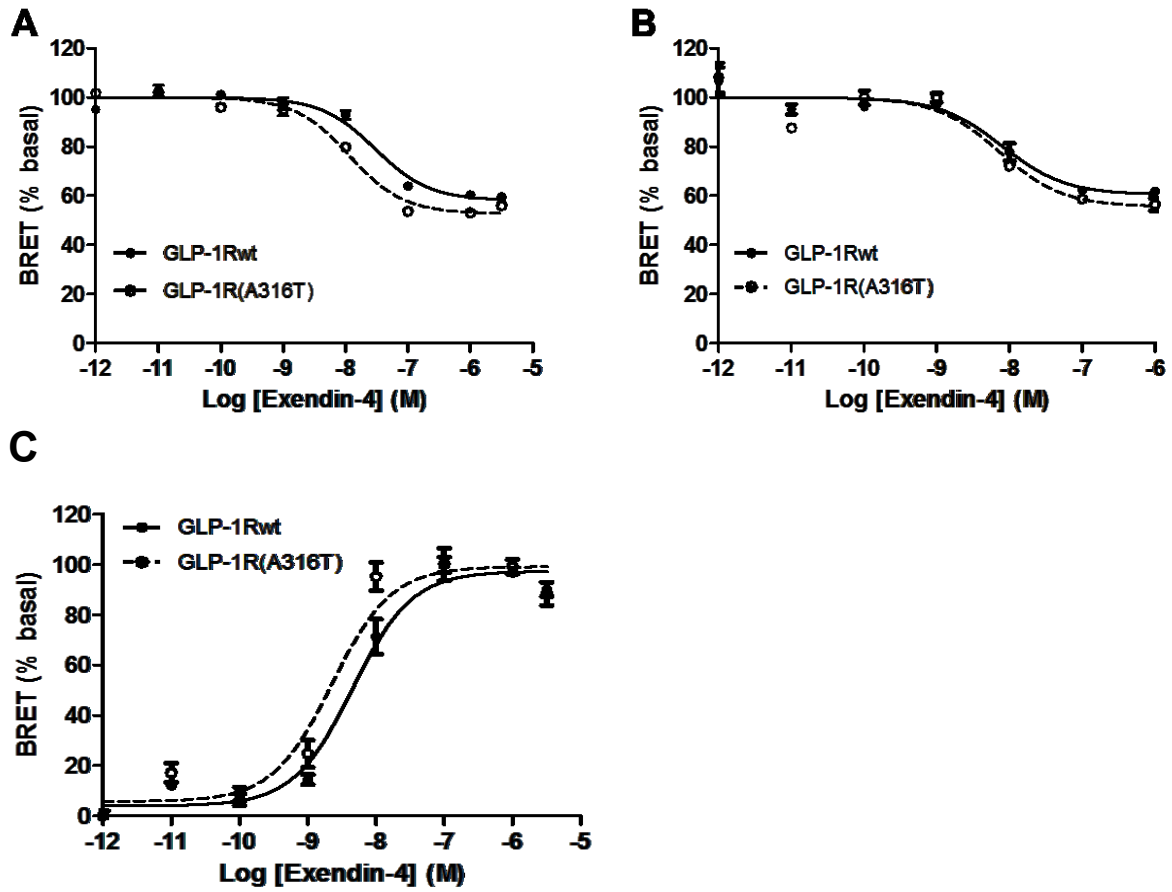

Figure 22. Interaction between GLP-1R (wt or A316T) and either rGFP-CAAX (plasma membrane marker) or rGFP-FYVE (early endosomal marker) upon exendin-4 stimulation. **(A)** BRET concentration-response curve assessing the agonist-stimulated GLP-1R-RLucII (wt and A316T) internalization using rGFP-CAAX BRET biosensor in Flp-In T-REx 293 cells. **(B)** BRET was measured in 1.1B4 cells. **(C)** BRET concentration-response curve assessing the agonist-stimulated GLP-1R-RLucII (wt and A316T) localization to early endosomal located rGFP-FYVE in Flp-In T-REx 293 cells. In all experiments, BRET was measured 30 min following addition of Exe-4. Data are expressed as net BRET ratio, represent the mean ± SEM of a typical experiment that was performed independently at least three times.

No difference in BRET intensity was detected between GLP-1R wt or GLP-1R (A316T), suggesting that the both receptors respond similarly to agonist stimulation by moving to the early endosome.

2.3 Discussion

Drugs approved by the U.S. Food and Drug Administration (FDA) must be safe, which indicates that their benefits must be greater than the known risks, and effective. It means that drug developers should prove safety of new therapies during clinical trials which requires additional time and increases their cost. Instead, genetic approach can help to evaluate the possible drug side effects on much earlier stages of drug development. Naturally occurring human polymorphisms that affect the activity of a particular protein can be used to estimate the probable efficacy and toxicity of a drug targeting such protein [16, 17]. Functional exonic variants that mimic the potential drug effects will help to make the right choice of potential targets or even implication of new drug targets. For example, a loss-of-function (LoF) variant may be useful tool for studying and understanding the consequences of pharmacological inhibition of drug target while a gain-of-function variant may demonstrate the effects of reversing the down-regulation of a pathway involved in disease pathogenesis [14].

Recent genome sequencing studies have identified a large number of potentially functional genomic variants in six genes that encode current drug targets for treating T2D. These variants are currently being studied intensively to find their association with the drug action on the encoded protein in clinical trials in T2D and obesity. Using targeted exome sequencing, a low-frequency missense variant in GLP-1R was identified which was associated with lower fasting glucose and risk of T2D, mimicking the effects of GLP-1R agonist therapy [13]. This variant was recently also associated with lower risk of coronary heart disease [14].

In the current study, we have identified pharmacological differences in the signalling profile of naturally occurring human GLP-1R polymorphic variant rs10305492 using a series of *in vitro* assays. In response to treatment with GLP-1 or Exe-4, GLP-1R is believed to signal through G-protein-regulated pathways ($G\alpha_s$ and $G\alpha_q$) as well as through G-protein-independent pathways by engaging the scaffold proteins, β-arrestins (**Figure 23**). Both cAMP production and intracellular Ca^{2+} mobilization are critical to the incretin response [9], whereas β-arrestins have been shown not only to terminate receptor G protein coupling but also to initiate alternative signalling pathways such as MAPK pathways and several others resulting in insulin secretion in pancreatic β-cells [130]. We assessed the functional impact of the GLP-1R polymorphic variant (A316T) on these three major GLP-1R signalling pathways. The mutant GLP-1R (A316T) is expressed at the cell membrane although the expression was

greatly reduced relative to the wild type receptor. This finding confirmed previous *in vitro* studies on GLP-1R mutations, which reported that A316T variant had as ~75% reduced cell surface expression compared with wild type, with no alteration in agonist binding affinity [186, 187].

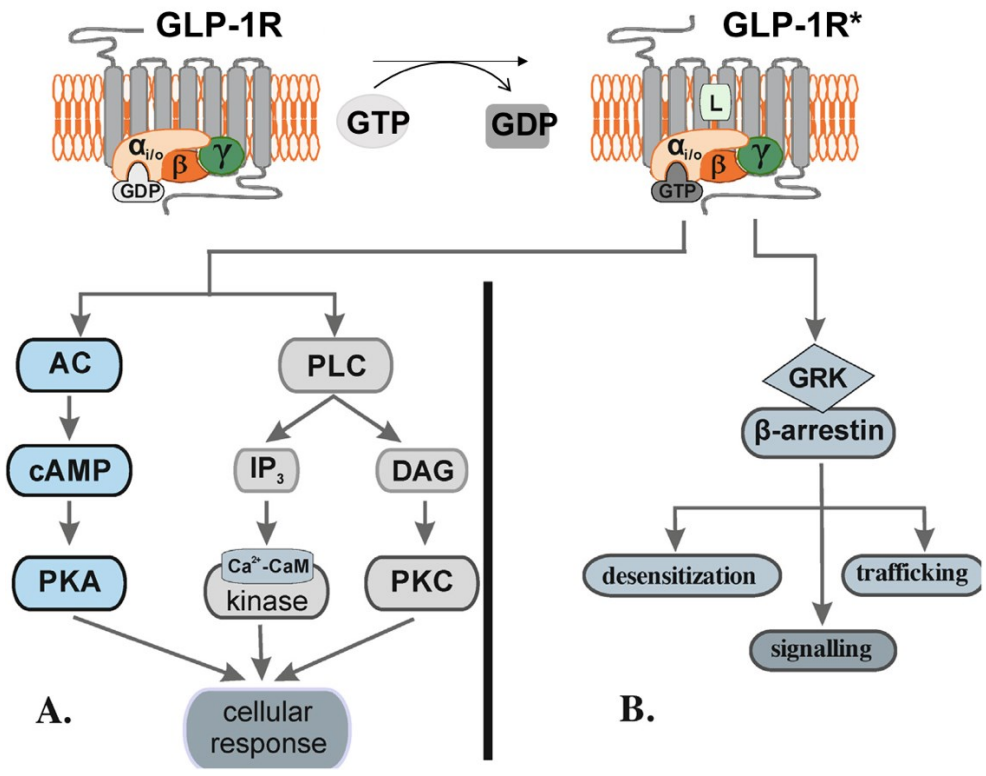

Figure 23. G-protein-dependent (A) and G-protein-independent (B) GLP-1R trafficking. After ligand binding GLP-1R activates downstream signalling through G-protein-dependent mechanisms ($G\alpha_s$ and $G\alpha_q$) as well as through G-protein-independent mechanisms by engaging β-arrestins and GRKs. Adapted from [213].

When intracellular Ca^{2+} mobilization in response to GLP-1R (A316T) activation ($G\alpha_q$ pathway) was assessed by measuring PKC activity, the mutant receptor showed decreased PKC activation. This finding is consistent with a previous report from Koole et al. [187] who also demonstrated that A316T mutation greatly reduced intracellular calcium mobilization in response to GLP-1 and Exe-4. Given that calcium mobilization induced by GLP-1R is a key factor in the early phase of insulin secretion by pancreatic β-cells, the reduced Ca^{2+}

mobilization is consistent with the reduced early insulin response observed for this variant reported by Wessel et al. [13]. It is also supported by another study where GLP-1R-knockout mouse was reported to have lower early insulin secretion relative to wild-type mice [214].

Apart from G-proteins, several other proteins are known to interact directly with GPCRs and regulate their activity. These include GRKs, which specifically recognize and phosphorylate the activated receptor, promoting recruitment of cytosolic β-arrestins [215]. In turn, the interaction between the GPCR and β-arrestins sterically prevents further interaction between the GPCR and G proteins and thus terminates G protein-dependent signalling [216]. Upon binding to an activated receptor, β-arrestins can also initiate alternative signalling pathways by acting as a scaffold protein [217]; and can also interact directly with clathrin-coated pit components and thereby mediate internalization of the receptor [218, 219].

β-Arrestin interaction with GLP-1R has received increased attention in recent years [126]. These scaffold proteins stabilize high-affinity conformations of GPCRs, which has led to the proposal of an alternative ternary complex model in which β-arrestins can substitute for G proteins [220]. Previous reports have demonstrated that β-arrestins are important for potentiating glucose-stimulated insulin secretion after GLP-1R stimulation in cultured pancreatic β-cells [7, 211]. Thus, the loss of β-arrestin signalling should result in impaired insulin secretion. In our studies, we have shown that the mutant GLP-1R(A316T) exhibited severely blunted β-arrestin recruitment when compared with the wild type receptor.

In a recent study, β-arrestin 2 was shown to interact with GLP-1R by two different mechanisms: a low-affinity phosphorylation-independent and a high-affinity phosphorylation-dependent pathway [212]. In the phosphorylation-dependent mechanism, GRK2 is initially recruited to the activated GLP-1R and β-arrestin 2 competes with GRK2 only after GRK2 recruitment and subsequent phosphorylation. Considering the important role of GRK2 in β-arrestin 2 recruitment, we used a BRET2 assay to study the effect of the kinase on the mutant receptor; and measured agonist-induced β-arrestin 2 recruitment to the GLP-1R with and without co-expression of GRK2. We showed that co-expression of GRK2 enhances β-arrestin 2 recruitment to the activated human GLP-1R increasing the affinity of the receptor toward glucagon, low-potency agonist on the GLP-1R (confirming the results of Jorgensen et al. [126]). However, in our hands, the mutant receptor failed to show any response to GRK2 overexpression confirming that the A316T mutation reduces interaction between GLP-1R and the β-arrestin signalling pathway.

As β-arrestin coupling is believed to play a key role in GPCR desensitization, we further investigated the effect of the mutation A316T on GLP-1R internalization using different BRET assays. Recent reports have shown the importance of GLP-1R internalization for its full functionality [131, 221]. Roed et al. [221] demonstrated that inhibition of GLP-1R internalization using a dynamin inhibitor resulted in significantly reduced GLP-1R stimulated cAMP accumulation. In a similar study, Kuna et al [131] suggested that GLP-1R is trafficked to the lysosome after internalization and receptor activation generates cAMP at the membrane and at endosomes, with both contributing to glucose stimulated insulin secretion. The mechanism of GLP-1R internalization is not completely understood and there are several studies showing that β-arrestin can regulate receptor signalling without affecting internalization [124, 132, 134, 171]. In our study, we found that both GLP-1R variants undergo rapid internalization in HEK 293. It supports studies by Syme et al. and Thompson et al. suggesting that caveolin-dependent GLP-1R internalization without involvement of β-arrestin is likely the most important mechanism [124, 134]. However, more detailed experiments should be performed to confirm these results.

Our *in vitro* pharmacological characterization of the mutant GLP-1R (A316T) revealed that despite low cell surface expression level the mutated receptor displayed comparable efficacy to the wild type receptor with regard to $G\alpha_s$ signalling. However, in contrast to the wild type receptor, the mutant GLP-1R has severely reduced ability to activate $G\alpha_q$ and β-arrestin pathways. Additional studies will be performed to evaluate both receptors at the same expression level to better determine the magnitude of these effects.

To explain these effects, we considered potential changes in GLP-1R conformation that could result from the A316T mutation, which is located at the interface of the third intracellular loop (IC_3) and transmembrane 5 domain (TM5). Because this receptor region is far removed from the ligand binding domains, it is unlikely that any changes in GLP-1R action observed at the A316T variant can result from a direct change in the hormone docking site. This conclusion is supported by our studies and others [187] where GLP-1R A316T mutant receptor has shown no changes in the GLP-1 or Exe-4 binding affinity. Wessel et al. [13] have proposed a structure for the mutant GLP-1R (A316T) based on a recently published structural model of the full-length human GLP-1R bound to Exe-4 [222]. In their model they proposed that the replacement of alanine by threonine at residue 316 of GLP-1R results in the disruption of hydrogen bonding between N320 (TM5) and E364 (TM6) by making a stable interaction with E364 (**Figure 24A**). This new intramolecular interaction can result in a slight

shift of TM5 towards the cytoplasm and of TM6 away from the cytoplasm, altering the conformation of the IC_3, which connects TM5 and TM6 within the cell (**Figure 24B**).

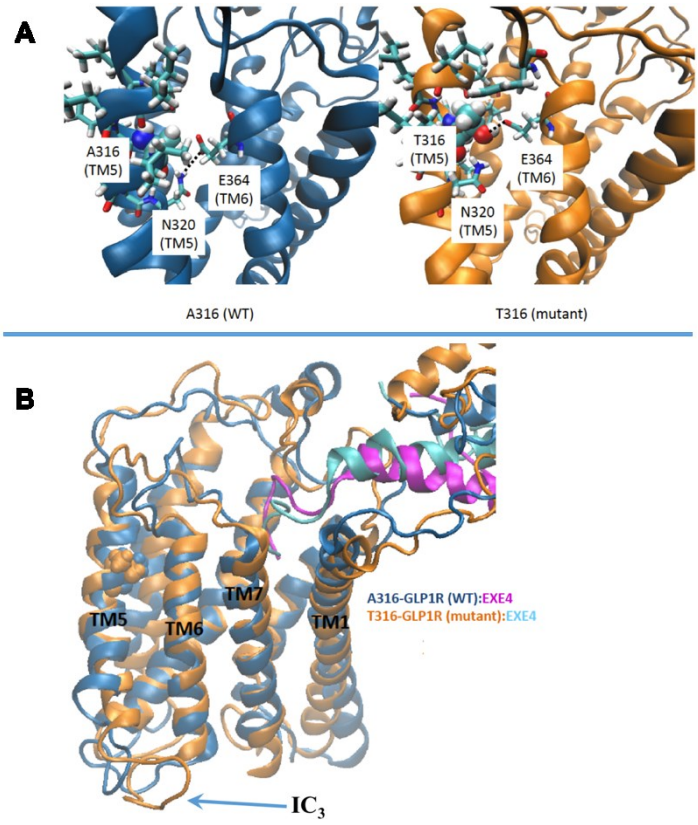

Figure 24. Comparison of structure conformation of human wild type GLP-1R and mutant GLP-1R(A316T). (**A)** In the wild type receptor (blue), residue N320 is supposed to interact with E364, whereas in the mutant receptor (orange) the T316 residue displaces N320 and takes its place. (**B**) The A316T mutation is proposed to alter the positions of the transmembrane domains of the mutant GLP-1R (A316T) bound to Exe-4, by shifting transmembrane domain 5 (TM5) slightly down towards the cytoplasm and TM6 slightly upward. The wild type receptor and Exe-4 are shown in blue and purple, respectively; while the mutant receptor and Exe-4 are shown in orange and cyan, respectively. Figure originally published in [13].

The importance of IC_3 in coupling GLP-1R to G-proteins is well documented [89, 90, 92]. For example, using a mutational scanning study and synthetic peptides with the sequences derived from the predicted intracellular loops of GLP-1R that mimic the effects of receptor

activation, Bavec et al. have suggested that the IC_3 loop is possibly the main switch which mediates signalling *via* GLP-1R to G-proteins, while the IC_1 and IC_2 loops function as signal modulators and might be important in discrimination between different types of G-proteins recruited by the receptor [223]. We suggest that a major effect of the A316T mutation is to stabilize the receptor conformation that induces signalling bias by activating differentially with intracellular signalling pathways (i.e. by favouring $G\alpha_s$ over other pathways). This conclusion disagrees with a previous suggestion by Wessel [13] that the A316T mutation activates the receptor in a constitutive manner, causing β-cells to secrete insulin at a lower glucose level, thereby maintaining a lower fasting glucose level (FG). In our experiments using BRET assays, we found no evidence that the mutant GLP-1R (A316T) had any constitutive activity *in vitro*.

Attenuated β-arrestin recruitment has been shown to reduce the insulinotropic properties of GLP-1R, which can explain the reduced early insulin secretion observed for this variant. In our opinion, increased glucoregulatory efficacy of the mutant GLP-1R (A316T) could not be attributed to an enhanced insulinotropic effect, as our data suggests that the mutant's ability to activate insulin secreting pathways is decreased compared to wild type. There must be an additional and distinct mechanism that might contribute to the improving of glycaemia. In accordance with this suggestion, a new biased GLP-1R peptide agonist P5, described by Zhang et al. [210], has been shown to attenuate β-arrestin recruitment while maintaining G-protein signalling. However, this agonist, despite having markedly lower insulinotropic properties, could induce adipogenesis and was more effective at correcting hyperglycaemia in diabetic animals than exendin-4, confirming the potential utility of biased agonists as novel GLP-1R therapeutics.

2.4 Conclusions

Our experiments attempted to determine the functional consequences of naturally occurring GLP-1R coding variants on the signalling properties of the receptor. As part of our studies, we also developed and validated a new strategy for assessing the GLP-1R cell membrane expression level using FACS and molecular biology methods. By characterizing the pharmacological consequences of this clinically relevant mutation, we have identified several mutation-induced changes in receptor function, which involve reduced receptor expression on the cell surface as well as diminished agonist-stimulated signalling responses *via* two distinct

pathways, $_{i}Ca^{2+}$ mobilization and β-arrestin recruitment. Detailed knowledge of the effects of the A316T mutation on the pharmacological properties of the GLP-1R can help in the design of new drugs that will specifically target the certain signalling pathway. Indeed, a new selective GLP-1R G-protein agonist P5 has recently demonstrated a preference for promoting GLP-1R-mediated G-protein activation over β-arrestin recruitment (referred to as G-protein bias) [210]. A direct comparison of P5 action *in vivo* with Exe-4 revealed that G-protein signalling downstream of receptor activation in the absence of β-arrestin signalling is sufficient to correct hyperglycemia, improve insulin sensitivity, preserve pancreatic islet integrity and improve liver steatosis [210]. The authors have demonstrated that loss of β-arrestin signalling after GLP-1R stimulation with P5 resulted in impaired insulin secretion in β-cells, whereas it promoted hyperplasia in epididymal white adipose tissue (eWAT) and reduced plasma adipokine levels. Thus, with respect to insulin secretion, mutation-induced changes in coupling to Ca^{2+} mobilization and β-arrestin may be more clinically relevant, suggesting that it may be pertinent to develop T2D therapies that differentially modulate these pathways in favor of cAMP accumulation.

CHAPTER 3. Future Directions

Two key considerations in assessing the possibility for genetic variants to be used in understanding therapeutic effects are that 1) the variant completely mirror the effects of drug action on the same target; and 2) knowledge of the functional consequences of the variant is known at an appropriate level of molecular detail. Genetic association data, reported previously [13, 14] suggest that the rs10305492 minor allele in GLP-1R is associated with reduced T2D susceptibility, lower fasting glucose levels, reduced insulin response to a glucose challenge and higher 2-h postprandial glucose levels. GLP-1R agonists are used clinically to reduce FG levels in patients with T2D [224]; and GLP-1R knockout mice show fasting hyperglycemia [214, 225]. Based on these findings, we proposed that the glucose-lowering rs10305492 allele confers gain of function to the mutant GLP-1R [13]. However, the functional effects of this variant in cellular models could not explain how this gain of function changed the signalling properties of GLP-1R [186, 187].

Previous studies of the changes in the GLP-1R signalling pathways have demonstrated that $_{i}Ca^{2+}$ signalling was dependent on the cell surface expression and also it was found to be much more sensitive to conformational changes in the receptor structure [187]. The present work is the first detailed study of the functional effect of the rs10305492 mutation on GLP-1R function. We show that even though this mutation reduces GLP-1R expression on the cell membrane, the mutated receptor has the same cAMP activity and similar internalization properties as a wild type receptor. However, β-arrestin recruitment and $G\alpha_q$- protein interaction were significantly reduced in the mutant receptor. Taken together, our results suggest that the replacement of Ala^{316} by Thr induces conformational changes in the receptor that differentially disturb receptor coupling to downstream pathways: these changes favor binding to the $G\alpha_s$ protein and greatly diminish binding affinity for $G\alpha_q$- and β-arrestin proteins resulting in biased signalling. Considering the lower cell membrane expression of the mutant GLP-1R (A316T), future experiments will be needed to confirm our hypothesis.

The phenomenon of biased agonism describes the ability of different ligands of the same receptor to promote distinct cellular responses [226]. Biased signalling is currently attracting great interest in both biological and therapeutic settings, as it provides a potential approach to specifically target cellular responses which favor therapeutically beneficial signalling

pathways over those leading to harmful effects. However, the mechanistic basis underlying biased signalling should be completely understood before using in rational drug design.

Pleiotropic coupling of GLP-1R leads to cAMP production, Ca^{2+} mobilization, and phosphorylation of ERK1/2 (pERK1/2), all of which are believed to be physiologically important [9, 11]. The contribution of these downstream effects and the extent to which one is activated relative to another is therefore crucial for optimal drug development. Several published studies have demonstrated that biased signalling does occur at the GLP-1R; however, the mechanism of this effect is still not completely understood [10, 11, 227-229]. These results emphasize the significance of our finding of a significant decrease in β-arrestin recruitment after mutant receptor stimulation is becoming of big importance. Genetic studies have shown that mutant GLP-1R (A316T) is associated with low FG and lower insulin response to a glucose challenge. It is generally accepted that β-arrestin signalling downstream GLP-1R activation plays a critical role in potentiating glucose-stimulated insulin release and that this effect may be influenced by genetic variations in the receptor. However, current genetic models for common diseases like T2D are based on the expectation that common mutations in GLP-1R would mildly impair receptor function and decrease insulin secretion: in this context it would be crucial to explain the beneficial action of the A316T mutant.

Interestingly, some of the effects of the A316T mutation can be observed for a recently developed GLP-1R peptide agonist (called P5), which exhibited blunted β-arrestin response while maintaining G-protein signalling [210]. This ligand was shown to be more effective at correcting hyperglycemia in diabetic mice than Exe-4, despite having markedly lower insulinotropic properties. Based on these findings, Zhang et al. have suggested the existence of another mechanism for improving glycemia by this ligand that is distinct from the insulinotropic effect. They have shown that P5 treatment induced changes in adipose tissue in mice, significantly increasing the number of small adipocytes and decreasing adipose tissue inflammation and insulin resistance. Their findings confirmed previous studies of non β-cell actions of GLP-1R agonists in regulating glucose metabolism [230-232]. The key mechanism for P5-induced glycaemic control in adipocytes was attributed to changes in the expression of peroxisome proliferator-activated receptor gamma (PPARγ) [210], a nuclear receptor that is responsible for increasing the expression of genes that stimulate adipogenesis and improve insulin sensitivity (e.g. Glut4, CD36). Based on this data, Zhang et al. proposed that the GLP-1R agonist P5 can preserve pancreatic β-cell function while minimizing the insulin secretory burden by promoting adipocyte hyperplasia and restoring insulin responsiveness, resulting in

better long term glycaemic control [210]. These findings point to the importance of conducting functional studies of mutant variants of GLP-1R in a broader range of tissues and cells, particularly in light of growing evidence of indirect regulation of glucose metabolism by GLP-1 *via* GLP-1R expressed in adipocytes and peripheral and central neurons [68, 69].

Our studies did not examine an additional complicating factor in understanding and determining the structure and function of family B GPCRs, namely their tendency to form homo- and heterodimers. For example, the full potency of the secretin receptor has been shown to be dependent on the dimerization mediated by the lipid-exposed face of TM4 domain [177]. Disruption of the interface *via* mutation or co-incubation with TM4 peptides resulted in an 18- to 28-fold reduction in the ability of secretin to stimulate cAMP, without any change in ligand binding affinity [177]. A similar dimerization interface has been identified for the calcitonin receptor [176].

GLP-1R has also been shown to form homodimers with TM4 providing the primary dimerization interface [175]. Deep functional characterization has revealed that the disruption of GLP-1R dimerization has selective effects on receptor signalling with greater loss of $_{i}Ca^{2+}$ signalling relative to the changes in cAMP or pERK1/2 pathways, which could be explained by differential changes of coupling efficiency of GLP-1R to $G\alpha_s$, $G\alpha_q$ and β-arrestin [175]. In addition to forming homodimers, the GLP-1R can form heterodimers with other family B GPCRs. Interestingly, the heterologous coexpression of the GLP-1R with the gastric inhibitory polypeptide receptor (GIPR) has been shown to result in decreased $_{i}Ca^{2+}$ mobilization and β-arrestin recruitment [173]. While heterodimerization between GLP-1R and the glucagon receptor (GCGR) has also been described [233], there is still a debate about the impact of the cross-talk between these receptors. Considering that GLP-1R, GIPR, and GCGR are all involved in blood glucose regulation and are all expressed in pancreatic islets and adipose tissue, heterodimerization may provide an additional mechanism of ligand-induced signal bias. Thus, it would be interesting to examine whether the mutant GLP-1R is able to heterodimerize, and whether this has any impact on the recruitment of interacting proteins. If the mutation can promote the formation of new pharmacologically distinct hetero- or homo-dimers, it may be possible that these display different signalling capacities.

From this point of view, additional experiments can be performed:

a. ***Assess the functional effects of the Ala316Thr mutation in isolated mouse and human islets.*** The functional assays that have been performed for characterizing the mutant GLP-

1R (A316T) were carried out in cell lines such as CHO, HEK293 or MIN6. Therefore, it would be of interest to examine whether these effects similarly occur in primary mouse or human tissues. Specifically, expression of the endogenous form of the GLP-1R may be silenced by adenovirus-encoded shRNAs, prior to overexpression of the mutant. Insulin secretion and gene expression as well as the β-cells response to agonist stimulation under conditions of inflammatory or metabolic stress can be examined.

b. ***Assess the impact of GLP-1R (A316T) on insulin secretion.*** As mentioned earlier in this chapter, it is important to evaluate the insulin secretagogue activity of the mutant receptor in insulin-secreting cells. Insulin content in lysates of Ins1, MIN6 or 1.1B4 cells or primary β-cells, transduced with mutant or wild type receptors can be determined at baseline and following glucose stimulation using an insulin ELISA. In addition to measuring insulin content, expression of several functional β-cell markers (e.g. proinsulin, glucokinase, Glut2, PDX1), can be evaluated.

c. ***Assess the heterodimerization of GLP-1R (A316T) with GIPR and GCGR using BRET techniques.*** BRET assays are a generally accepted technique for assessing protein-protein interaction as it allows the study of the receptors cross-talk temporally and spatially in living cells. Using BRET, ligand or glucose stimulatory effects on GLP-1R-protein interactions can also be assessed in real time.

d. ***Assess the effect of the Ala316Thr mutation on apoptosis and proliferation of pancreatic cell lines (Ins1, 1.1.B4 or MIN6) and primary β-cells.*** As mentioned previously, GLP-1R activation not only enhances insulin secretion but also promotes proliferation and inhibits apoptosis of pancreatic β-cells. Thus, it would be of interest to examine how this mutation may affect these two parameters using cell lines or primary tissue. Viability assays, such as the MTT assay can be used.

e. ***Assess the effect of GLP-1R (A316T) in stressed pancreatic β-cells.*** In addition to measuring the mutant impact on insulin secretion and β-cell gene expression under basal conditions, it is important to test its effect under conditions of prolonged exposure to cytokines (inflammatory stress) as well as prolonged exposure to elevated levels of glucose and fatty acids (metabolic stress).

f. ***Assess the effect of GLP-1R (A316T) on adipogenesis.*** Since GLP-1 and GLP-1R promote adipogenesis through the activation of PPARγ expression, it would be of the

interest to evaluate the effects of the mutant GLP-1R in 3T3-L1 cell preadipocyte differentiation and adipocyte responsiveness to insulin.

In conclusion, we hypothesized that the rs10305492 variant associated with lower T2D risk induces signalling bias by stabilizing a GLP-1R conformation that couples differentially to intracellular signalling pathways compared to the wild-type receptor. The future functional studies proposed above will further characterize the action of the mutant GLP-1R in a broader range of tissues and cells to explain the beneficial action of the A316T mutant. Furthermore, our studies characterizing function of the A316T mutant GLP-1R using the panel of BRET biosensors, have confirmed the validity of the BRET approach to evaluate the functional effects of genetic variants in GLP-1R and GPCRs in general. This approach will become particularly valuable in the near future, when large exome sequencing projects reveal increasing numbers of variants in GLP-1R and other GPCRs associated with T2D susceptibility (or that influence physiological functions of cells in the pancreatic islets and insulin-sensitive tissues) that may alter the signalling properties of these GPCRs and contribute the pathogenesis of T2D or its cardiometabolic complications.

Abbreviations

cAMP	cyclic adenosine monophosphate
ATP	adenosine triphosphate
AT1R	angiotensin II type 1 receptor
AC	adenylyl cyclase
BRET	bioluminescence resonance energy transfer
BAD	Bcl2-associated death promoter
CICR	calcium-induced Ca^{2+} release
CNS	central nervous system
CHD	coronary heart disease
CRE	cAMP response elements
CREB	cAMP-response-element-binding protein
DAG	diacylglycerol
DDP-IV	dipeptidyl peptidase IV
DMEM	Dulbecco's modified Eagle's medium
ECL	extracellular loop
EGFR	epidermal growth factor receptor
EPAC	Exchange Protein directly Activated by cAMP
ER	endoplasmic reticulum
ERK1/2	extracellular signal-regulated kinase1/2
ExAC	Exome Aggregation Consortium
Exe-4	exendin-4
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FDA	United States Food and Drug Administration
FG	fasting glucose
FI	fasting insulin
FoxO1	forkhead box protein O1
GCG	glucagon coding gene
GCGR	glucagon receptor
GEF	guanine nucleotide exchange factors
GFP	green fluorescent protein
GIP	gastric inhibitory peptide
GIPR	gastric inhibitory polypeptide receptor
GLP-1	glucagon-like peptide-1
GPCRs	G-protein coupled receptors
GRK	GPCR kinase
GRPP	Glincentin-related polypeptide
GSIS	glucose-stimulated insulin secretion
GTPases	GTP-binding proteins
HTS	high-throughput screening
ICL	intracellular loop
IP	intervening peptide
IP_3	inositol-1,4,5-triphosphate
IRS-2	insulin receptor substrate 2
IRP	immediately releasable pool
MAF	minor allele frequency
MAPK	mitogen-activated protein kinases

MPGF	major proglucagon fragment
NF-κB	nuclear factor κB
PAM	positive allosteric modulators
PC	proprotein convertases
PDX-1	pancreas duodenum homeobox-1
PI_3K	phosphoinositide 3-kinase
PIP_2	phosphatydilinositol-4,5-biphosphate
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PLC	phospholipase C
PPARγ	peroxisome proliferator-activated receptor gamma
RLuc	Renilla reniformis luciferase
RP	reserve pool
RRP	readily releasable pool
SNP	single nucleotide polymorphisms
SUMO	Small ubiquitin-related modifier protein
T2D	type 2 diabetes mellitus
TM	transmembrane domains
VGCC	voltage-gated Ca^{2+} channels
eWAT	epididymal white adipose tissue
YFP	yellow fluorescent protein

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