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New insights into beta cell GLP-1 receptor and cAMP signaling

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Abstract

Harnessing the translational potential of the GLP-1/GLP-1R system in pancreatic beta cells has led to the development of established GLP-1R-based therapies for the long-term preservation of beta cell function. In this review, we discuss recent advances in the current research on the GLP-1/GLP-1R system in beta cells, including the regulation of signaling by endocytic trafficking as well as the application of concepts such as signal bias, allosteric modulation, dual agonism, polymorphic receptor variants, spatial compartmentalization of cAMP signaling and new downstream signaling targets involved in the control of beta cell function.

Introduction

Despite human islets expressing almost three hundred G protein-coupled receptors (GPCRs), the only islet GPCR for which agonists are in current clinical use is the glucagon-like peptide 1 (GLP-1) receptor (GLP-1R) [1]. Almost forty years after the discovery of the GLP-1 sequence from cDNA, which unveiled that it was encoded by the larger preproglucagon precursor [2], followed by the discovery and cloning of the GLP-1R by the Thorens group in 1992 [3], the enormous translational potential of the GLP-1/GLP-1R system is now evident, with GLP-1R-based therapies recognized to improve a number of clinical parameters in patients with type 2 diabetes mellitus (T2DM) while being associated with a low risk of hypoglycemia based on their strictly glucose-dependent insulinotropic action. Consequently, these incretin-based therapies have become an established option for the long-term preservation of beta cell function [4].

The complexity of signaling mediated by the GLP-1R [5] continues to be the source of intense ongoing investigations. Advances in recent years include the application of concepts such as signal bias, allosteric modulation, dual agonism and the study of polymorphic receptor variants to the GLP-1R (for a summary of past investigations, see [6]). Additional recent areas of research include spatial compartmentalization of cAMP signaling as well as

the identification of new targets downstream of cAMP generation to control beta cell function.

Signal bias at the GLP-1R

An idea gaining traction for the development of new GLP-1R-targeting therapeutic approaches is based on the concept of signal bias, which describes the ability of ligands for the same GPCR to trigger distinct patterns of intracellular signaling by preferentially stabilizing specific active conformations of the receptor [7]. In the case of the GLP-1R, two distinct domains have been identified to control peptide-mediated biased agonism [8]. Changes in the spatial orientation of transmembrane helices (TMs) 5 and 6 and restructuring of the extracellular loop (ECL) 2 are critical for the propagation of signals related to cyclic adenosine monophosphate (cAMP) generation and elevation of intracellular Ca^{2+}, while re-ordering of TMs 1 and 7 is required for extracellular signal-regulated kinase (ERK) 1/2 activity. In this context, backbone modifications of the natural GLP-1R agonist (GLP-1RA), GLP-1, via the replacement of selected amino acid residues, have been used to generate GLP-1 analogues with distinct preferences for promoting G protein activation *versus* β-arrestin recruitment [9]. Moreover, a recent study found that single amino acid changes at the amino-terminal end region of the pharmacologically stable GLP-1RA exendin-4 resulted in pronounced signal bias and modulation of GLP-1R endocytic trafficking. In this study, ligands that retain the receptor at the plasma membrane displayed reduced β-arrestin recruitment and faster agonist dissociation rates, and produced greater long-term insulin release without concomitant increases in nausea (the common side effect of GLP-1R therapies [10]). These results have recently been confirmed in a further study showing that GLP-1R internalization can control the spatiotemporal profile of signaling mediated by biased agonism [11]. One potential caveat to this approach is that agonists that associate with increased preservation of cell surface receptors via reduced receptor internalization and/or increased recycling appear to display concomitant reductions in efficacy and potency, such that beyond a certain bias threshold, glucose regulation does not continue to increase.

A further consideration would be the possible divergent effect of bias in the different tissues where the GLP-1R is expressed. Further elucidation of the contribution of trafficking pathways to the potentiation of beta cell function relative to the prevalence of side effects will be important for the optimal development of novel GLP-1R-based therapeutics.

Spatiotemporal control of GLP-1R signaling

The abovementioned studies, in addition to work from the wider family of GPCRs [12], and other membrane receptors [13], have led to the hypothesis that the regulation of endocytic trafficking pathways can be exploited to achieve a higher level of spatiotemporal control of GLP-1R signaling, particularly since the realization that GPCR signaling is not only confined to the plasma membrane, but can occur at variable intracellular locations [14], indicating that receptor endocytic trafficking might not only provide an adaptable system to titrate cellular signaling and maintain homeostatic regulation, but can specifically determine cellular responses by altering the location and duration of specific receptor-mediated signaling pathways [12]. In the case of the GLP-1R, the receptor is known to undergo rapid (half-time of 2-3 minutes) clathrin-dependent [15], but arrestin-independent [16], endocytosis following GLP-1 exposure, with receptor recycling occurring with a half-time of around 15 minutes and progressive accumulation of the receptor in endosomal compartments [15], where prolonged association of the G protein alpha subunit Gαs with activated receptors has been found to contribute to sustained cAMP generation [17]. Based on previous studies that have identified key endocytic players involved in the control of GPCR trafficking and signaling [18-20], a screen for major endocytic regulators of the GLP-1R in pancreatic beta cells was recently performed [21]. In this study, the sorting nexins SNX1 and SNX27, known endosomal regulators of receptor recycling *versus* degradation, were identified as key factors for the control of beta cell responses to GLP-1R agonism. SNX27 is required to sustain GLP-1R recycling and resensitization while also inhibiting GLP-1R endosomal signaling, while SNX1 is required to restrict the rate of GLP-1R recycling. Additionally, huntingtin interacting protein 1 (HIP1), a factor involved in Huntington's disease known to control the level of endocytosis

and signaling of other membrane receptors [22, 23], was also found to be important in the coordination between GLP-1R endocytosis and desensitization at the plasma membrane. Once activated and desensitized, the fraction of GLP-1Rs trafficked back to the plasma membrane via recycling pathways will determine the rate of resensitization of the receptor. This rate is considered to be dependent on receptor internalization, endosomal acidification and receptor recycling. Resensitization has also been recently shown to be regulated by endothelin-converting enzyme-1 (ECE-1) activity, most likely through the proteolysis of GLP-1 in endosomes which facilitates GLP-1R dephosphorylation and recycling [24].

GLP-1R structure and conformational dynamics

New advances in GPCR structural biology provide unprecedented insights into ligand binding, conformational dynamics and the control of signaling outcomes. Recently, a number of GLP-1R structures have been published – these include the crystal structures of the human GLP-1R transmembrane domain in complex with two different negative allosteric modulators [25]; an X-ray crystal structure of the full-length receptor bound to a truncated peptide agonist [26]; a cryo-EM structure of the full-length receptor bound with GLP-1 and coupled to Gαs [27]; and a cryo-EM structure of a biased agonist-bound GLP-1R-Gαs complex [28]. It is however important to consider that crystal structures of isolated receptor regions may not give a complete reflection of peptide-receptor interactions occurring in the dynamic, native, full-length receptor state, as demonstrated by mapping the interactions of exendin-4 with the full-length functional human GLP-1R using genetically-targeted photocross-linking in mammalian cells [29].

A complementary approach using molecular dynamics simulations with hybrid models of the full-length active and inactive receptors has unveiled a mechanism for agonist activation of the GLP-1R [30]. In this study, the GLP-1 His-7, Glu-9 and Asp-15 residues act together to destabilize TM6 and ECL3 in order to generate an active conformation of the GLP-1R. Additionally, a recent investigation into the transition path of the GLP-1R extracellular

domain (ECD) by molecular dynamics simulations and Markov state modeling [31] has shown that the ECD of the GLP-1R adopts a closed conformation in the GLP-1-bound state and opens in the GLP-1-unbound apo-state of the receptor. Using a combination of alanine-scanning mutagenesis and pharmacological quantification of the effects of mutations on peptide binding affinity and downstream signaling, key elements of the GLP-1R ECD involved in biased signaling responses engaged in a ligand-dependent manner have been identified [32]. Moreover, the same group has recently shown that endogenous variants of GLP-1 display differential engagement of polar networks within the GLP-1R [33], leading to the differential signal bias exhibited by these peptides. Finally, either homo- [34] or hetero-dimerization with other closely related class B GPCR members [35] is another potential mechanism by which the GLP-1R might regulate agonist signaling.

Role of GLP-1R signaling in beta cell identity

Another recent key area of research has concentrated on the role of GLP-1R in promoting beta cell identity. Previous studies have shown that alpha cells, especially those at earlier stages of differentiation as well as alpha progenitor cells, might be the precursors of beta cells. Treatment with GLP-1 in rats exposed to a single high-dose of streptozotocin (STZ) to induce beta cell death results in increases in cell populations positive for both insulin and glucagon (GCG), suggesting that GLP-1R signaling might promote beta cell neogenesis by the trans-differentiation of alpha cells [31]. The proposed mechanism involves the regulation of the PI3K/Akt/FOXO1 pathway, resulting in increased pancreatic and duodenal homeobox 1 (Pdx1) and MafA expression but decreased MafB expression. In a separate study, new beta cells originating from non-beta cells were found to be significantly increased in mice infected with GLP-1-expressing adenoviruses under the control of the rat Ins2 (RIP) promoter. Increased numbers of insulin-producing, GCG-positive cells were observed through a mechanism that required increased expression and secretion of fibroblast growth factor 21 (FGF21) [36]. On the other hand, a study in a novel alpha-cell-specific GLP-1R knockout mouse model has demonstrated that absence of alpha cell GLP-1Rs does not

interfere with beta cell function but affects GCG secretion in a glucose-dependent bidirectional manner [31], suggesting a direct role of the receptor on alpha cell function.

Much of the research on the role of the GLP-1R in the promotion of beta cell expansion has been carried out in rodents or using *in vitro* culture models. Surrogate measurements of beta cell mass in humans suggest that the promising effects of GLP-1 analogues to promote beta cell proliferation and protect against apoptosis in rodents might not occur in humans. A mechanism by which exendin-4-induced GLP-1R activation might induce human beta cell proliferation within juvenile, but not adult, islets was recently suggested to involve calcineurin/nuclear factor of activated T cells (NFAT) signaling in a study with human islets grafted into immunodeficient mice [37]. The development of novel engraftment systems, including the recently developed intraocular *in vivo* imaging of pancreatic islets [38] will enable further assessments of human beta cell replication in humanized mouse models that might eventually provide new avenues to achieve functional beta cell expansion in humans.

Intra-islet GLP-1

Increasing evidence suggests that GLP-1 can be produced locally within islets, predominantly by alpha cells [39], with alpha cell-produced GLP-1 shown to promote insulin secretion [40] and beta cell survival [41], as well as beta cell mass compensation [42] in the face of metabolic stress. In a study with human and mouse pancreatic islets, production of interleukin 6 (IL6) by alpha cells was apparently responsible for the intra-islet production of GLP-1 and insulin, with this process being regulated by inflammation [43]. In a separate study, the GLP-1R-specific antagonist exendin[9-39] was administered to mice with tissue-specific reactivation of the preproglucagon gene. These experiments showed that while exendin[9-39] had no effect on blood glucose in mice with restoration of intestinal preproglucagon, pancreatic reactivation of this gene fully restored the impairment of glucose tolerance caused by exendin[9-39]. These data suggest that islet-produced GLP-1 can play an important role in regulating glucose homeostasis [44]. Signaling from intra-islet

proglucagon-derived peptides was recently found to require both the GLP-1R and the GCG receptor (GCGR), with loss of these peptides decreasing insulin secretion via reduced beta cell cAMP generation and with input from alpha cells essential to maintain glucose tolerance under metabolic stress [45]. The extent to which alpha cell GLP-1 is required for the potentiation of insulin secretion under normal conditions remains to be ascertained, with some reports suggesting that it is in fact GCG that plays a prominent role in this process [46], as it is now thought to act as a as an insulinotropic hormone in the fed state [47].

Extra-pancreatic GLP-1R activity

The expression of the GLP-1R is not confined to islets but is also present in peripheral and central nervous systems, among other tissues. Activation of extra-pancreatic GLP-1R results in a reduction in gastric motility and a decrease in feeding (due to reduced appetite and increased satiety), as well as a decrease in hepatic glucose production, stimulation of brown adipose tissue (BAT) thermogenesis, decreased white adipose tissue (WAT) lipogenesis, increased glomerular filtration rates, changes in blood pressure and improved coronary flow [48], although the extent to which the latter effects are relevant to humans remains to be established (see Figure 1 for an overview of GLP-1 intra islet and extra-pancreatic actions).

It should also be noted that the effects of GLP-1 are often glucose-dependent and that some responses are mainly observed under pathophysiological (obesity, diabetes) states in response to pharmacological doses of GLP-1RAs. GLP-1 analogues currently used to treat T2DM have shown additional benefits for the treatment of obesity through hypothalamic signaling to reduce food intake and through the gut by delaying gastric emptying [49]. GLP-1RAs are also currently under investigation as treatments for a range of heart, liver, kidney, lung and brain related disorders [50, 51].

Highlighting the importance of extra-pancreatic GLP-1R signaling for its beneficial effects on glucose homeostasis, beta cell GLP-1Rs appear to be dispensable for maintaining glucose

tolerance after vertical sleeve gastrectomy (VSG) in obese mice, as shown in beta cell-specific GLP-1R knockdown mice [52]. Additionally, the beneficial effects of the GLP-1R pharmacological agonist lixisenatide on gastric emptying and insulin secretion leading to improved glycemia in diabetic mouse models were abolished in vagotomized mice, indicating the requirement of a functional gut-brain axis [53]. In this context, Glp1R0017 is a recently developed monoclonal blocking antibody to the GLP-1R that blocks the actions of GLP-1 *in vivo*, holding the potential to be used in the investigation of the physiological importance of GLP-1Rs in extra-pancreatic tissues where GLP-1R targets and downstream signaling pathways are poorly understood [54].

Polymorphisms of the *glp1r* gene

An increasing number of genetic variants for gastrointestinal hormones and their cognate receptors are currently being associated with obesity phenotypes, appetite regulation, and metabolic disturbances. The field of pharmacogenetics is rapidly expanding as a key element of precision medicine, with current research also focusing on responses to GLP-1RAs and other second-line antidiabetic medications [55, 56]. For the GLP-1R, a pilot study in nondiabetic subjects assessed the effect of receptor polymorphisms on insulin secretion in response to infused GLP-1, with two single nucleotide polymorphisms (SNPs), rs6923761 and rs3765467, associated with changes in responsivity to GLP-1 [57]. The GLP-1R Gly168Ser missense variant was also associated with reduced glycemic responses to dipeptidyl peptidase-4 (DPP-4) inhibitors (also known as “gliptins”), a treatment that acts by increasing endogenous incretin levels [58]. However, a further study in patients with poorly controlled T2DM found no statistical correlation between *glp1r* polymorphisms and GLP-1 responses [59]. Pharmacogenetic studies have additionally suggested a role for GLP-1R genetic variation in the extra-pancreatic effects of GLP-1RAs, including effects on gastric emptying [60] and cardiovascular safety of GLP-1RAs [61].

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Selected *glp1r* SNPs have also been studied for changes associated with ligand-binding and signaling alterations [62]. In the latter study, a major loss of GLP-1R function was found to be associated with the Thr149Met polymorphic receptor variant, previously reported as showing a connection with T2DM [63]. Moreover, attenuated responses to small molecule allosteric modulation of the receptor were associated with the Ser333Cys variant. These two variants were functionally characterized through replacement of the corresponding residues to a panel of alternate amino acids in a subsequent study [64]. This study highlighted the importance of these sites for the maintenance of GLP-1R G protein coupling and allosteric responsiveness, respectively. Additionally, association of low-frequency rare exome variants with lower fasting glucose [65] identified the GLP-1R Ala316Thr missense variant as being linked with reduced susceptibility to the development of T2DM. This variant was also independently associated with protection against coronary heart disease [61]. Additionally, GLP-1R genetic polymorphisms have been associated with increased T2DM and/or coronary heart disease risk in East Asian [66], Egyptian [67], Japanese [63, 68] and Chinese populations [69].

Dual and triple incretin peptides – "twincretins" and "tricretins"

GLP-1 mimetics are now well established as a treatment modality for T2DM and associated metabolic conditions, with ongoing interest in other areas such as cardiovascular disease [70], neurodegeneration [71], and raised intracranial pressure [72]. Recent innovative ideas to prolong the action of GLP-1RAs for the treatment of T2DM have included the generation of dimerized GLP-1 analogues via covalent linkage [73], and the generation of peptide-oligourea hybrid analogues of GLP-1 with improved action *in vivo* [74].

However, GLP-1R is one of many GPCRs coupled to insulin secretion in beta cells. Therefore, combined stimulation of more than one receptor target is a rational strategy to enhance the insulinotropic effect of anti-diabetic drugs. These efforts have primarily focused on receptor combinations with a high degree of homology to simplify agonist design, with the

glucagon receptor superfamily being the best studied example. The work using oxyntomodulin, a natural dual agonist for GLP-1R and GCGR, provides a blueprint for the potential therapeutic advantages of targeting both receptors simultaneously. Like GLP-1, oxyntomodulin stimulates insulin secretion by direct action on beta cells [75] and when administered systemically [76, 77], and suppresses appetite leading to weight loss [78][79]. However, oxyntomodulin also harnesses GCGR-mediated effects on whole body energy homeostasis, augmenting weight loss by increasing energy expenditure [80, 81]. Oxyntomodulin is a low affinity agonist for both receptors and has a short circulatory half-life, but optimization of its amino acid sequence by many groups has generated a plethora of pharmacokinetically viable synthetic dual GLP-1R/GCGR peptide agonists [82, 83], some of which have now demonstrated positive results in late stage clinical trials [84]. Whilst a significant component of their overall metabolic effect depends on GCGR-mediated increases in energy expenditure to increase weight loss [85, 86], recent studies highlight an important role for intra-islet GCG signaling in supporting insulin release [46]. This carries a caveat that the glucagon contribution to insulin secretion probably arises jointly from action at GCGR and GLP-1R, as glucagon is in fact a low affinity agonist at both human [87, 88] and mouse GLP-1Rs [46]. Indeed, the cAMP-stimulating and insulinotropic effects of glucagon can be partially blocked by the GLP-1R antagonist exendin(9-39) in mice and in INS-1 832/3 rat beta cells [46, 88]. However, data presented in the same studies using *gcg*$^{-/-}$ islets and the orthosteric GCGR antagonist des-his^{1}glu^{9}-glucagon demonstrated that intact GCGR signaling is required for full glucagon action on beta cells. These observations are corroborated by independent studies using the isolated perfused rat pancreas model [89] and when oxyntomodulin was administered to wild-type *versus* *gcgr*$^{-/-}$ mice under hyperglycemic clamp conditions, both of which found in favor of a direct insulinotropic effect of glucagon via its own receptor. Notably, a number of nominally GCGR-selective small molecule antagonists have been shown to also inhibit the action of both GLP-1 and glucagon at the GLP-1R [88], adding additional layers of complexity when interpreting studies using such compounds to understand GCGR actions [90]. In contrast, orthosteric

peptide probes (e.g. des-his^{1}glu^{9}-glucagon) appear to be GCGR-specific and are not subject to this caveat [46].

The administration of GLP-1R agonists in combination with agonists of the glucose-dependent insulinotropic polypeptide (GIP) receptor (GIPR), the other incretin receptor of importance for the regulation of beta cell function [91], has also been attempted, with additive contributions to postprandial glucose regulation in healthy individuals [92]. However, the well-established observation that patients with T2DM are insensitive to GIP [93], perhaps due to downregulated expression of the GIPR in the context of chronic hyperglycemia [94], has limited the therapeutic development of GIPR agonists. More recently, a number of studies have showed that GIP responsiveness can be restored by concomitant or prior treatment with another anti-hyperglycemic agent [95-98]. In this context, positive results for dual GLP-1R/GIPR peptide agonist LY3298176 have recently been reported [99, 100], outperforming the pure GLP-1R agonist Dulaglutide for both glycemic and body weight outcomes. Nevertheless, the GIPR contribution to the overall glucoregulatory effects of this molecule in humans are debated, as despite having a significantly lower GLP-1R potency than Dulaglutide, the dose of the dual agonist administered was significantly higher, meaning that equivalent GLP-1R occupancy may well have been achieved. Moreover, the weight loss effects of Dulaglutide are not class-leading for a pure GLP-1R agonist [101], presumably due to reduced CNS penetration due to the large size of the molecule. Semaglutide, which like LY3298176 relies on a fatty acid chain to extend pharmacokinetics by reversible albumin binding [102], would be a more appropriate comparator. Impressive preclinical results were seen with a triple GLP-1R/GCGR/GIPR agonist [103]. Unpicking the molecular mechanisms by which these compounds augment insulin secretion is complex, as contributions are possible from many sources, including interactions between each type of receptor within the plasma membrane [35], signaling pathway cross-talk [104] or simple accumulation of independent insulinotropic signals from each activated receptor. A peptide with activity at GLP-1R, GCGR and GIPR was found to activate a broader selection of G

proteins than GLP-1 itself [105], although it cannot be concluded that this depended on simultaneous activation of all three target receptors, as the tri-agonist itself was more insulinotropic in isolated islets than co-administration of equimolar doses of GLP-1, GCG and GIP.

As mentioned before, formation of heteromers within the glucagon receptor superfamily has been previously described [106], in some cases functionally impacting signaling and trafficking phenotypes. It has however not been unambiguously established whether this process occurs endogenously in pancreatic beta cells. Recent work has revealed characteristic differences in how incretin receptors segregate into plasma membrane nanodomains in beta cells [107]. This receptor segregation could influence the formation of functional receptor-receptor interactions under basal or agonist-stimulated conditions. Intriguingly, heterobivalent antibody Fc fusion ligands have been developed bearing peptide sequences to simultaneously target GLP-1Rs, GCGRs and/or GIPRs via different arms of the molecule [108]. It seems plausible that these could promote simultaneous engagement of both receptor types in a confined membrane region, resulting in a different pattern of activation to that of bifunctional but monovalent peptide ligands, but this possibility has yet to be systematically investigated.

Some examples of GLP-1 peptide hybrid ligands targeting the GLP-1R and other GPCRs with putative beta cell actions are given in Table 1. Note that, in some cases, beta cells may not be the exclusive or even the primary target of these ligands.

GLP-1R endocytosis as a mechanism to deliver therapeutic cargoes to beta cells

The rapid endocytic uptake of agonist-stimulated GLP-1Rs, along with their distribution mainly in the endocrine pancreas and central nervous system, has led to interest in the development of hybrid ligands which bind the GLP-1R specifically but do not depend therapeutically on canonical GLP-1RA actions such as insulin secretion *per se*. Instead,

these ligands make use of GLP-1R endocytosis to deliver a therapeutic “cargo” specifically to beta cells and potentially other GLP-1R-expressing tissues. The concept was arguably first developed for radioligands to localize and potentially treat GLP-1R-expressing neoplasms such as insulinomas [109]. Various GLP-1R ligands have been investigated, mostly derived from the exendin peptides and carrying radiotracers to allow whole body imaging using positron emission tomography (PET) or single photon emission computed tomography (SPECT) scanning, or to locally deliver cytotoxic radiotherapy to tumor cells. Whilst good selectivity for insulinoma cells is usually seen, a particular issue can be excessive uptake of radiotracer into the renal parenchyma via megalin-type receptors [110]. A variety of strategies have been employed, with varying degrees of success, to limit kidney exposure [111]. This issue should be borne in mind when using the GLP-1R as a purported specific entry point to pancreatic beta cells and other GLP-1R-expressing tissues.

A fascinating application, pioneered by Dimarchi, Muller and Tschöp, involves conjugation of various nuclear hormone receptor ligands to GPCR-targeting peptides, allowing them to realize their metabolic benefits in specific tissues without the side effects that usually limit their medical use. For example, coupling of thyroid hormone to a GCG mimetic ligand led to a robust hypocholesterolemic effect through the predominant activation of hepatic thyroid receptors without the usual cardiac or bone manifestations of hyperthyroidism [112]. Similarly, the GLP-1R has been exploited to deliver estrogen and the corticosteroid dexamethasone to islets and the brain [113, 114], without the adverse effects usually associated with their untargeted use. Whilst the metabolic effects of these “trojan horse” hybrid peptides are impressive, an unresolved mechanistic question concerns the exit route for the nuclear hormone component from the endosome into the cytosol towards the nucleus. Membrane estrogen receptors such as the G protein-coupled estrogen receptor (GPER), or membrane-associated estrogen receptors α and β, ordinarily contribute to cellular estrogen uptake [115], but their role in transporting estrogen liberated from its GLP-1 carrier peptide across the endosomal membrane has not been established, an important

mechanistical point that remains to be addressed, particularly when taking into account the estrogen receptor association with the cytoplasmic face of intracellular membranes. Sorting of the GLP-1R to intraluminal vesicles of multivesicular bodies following endocytosis [21] poses an additional level of complexity. Further studies will need to be performed to gain a fuller understanding of this process.

A recent study has extended this concept to the delivery of antisense oligonucleotides (ASOs) to pancreatic beta cells, using the GLP-1R to gain entry via clathrin-mediated endocytosis [116]. Employing a GLP-1 mimetic peptide coupled to an ASO targeting the widely expressed transcript metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), marked knockdown was achieved in beta cells without detectable effect in liver, which does not express the GLP-1R. The authors did not however report the degree of ASO exposure in kidney or brain, which would be important to consider in view of prior evidence indicating these locations as important sites of GLP-1 peptide uptake. A recurrent question remains concerning the mechanism of endosomal escape of the ASO once separated from the peptide ligand. In a different context, stepwise modulation of different components of the endolysosomal maturation pathway [117] revealed potential roles for Rab5C and Rab7A in the transport of ASOs to the cytosol following uptake via stabilin receptors. Whether this modulation reflects a specific transfer role or reflects an impact on the general integrity of the endocytic pathway is not obvious.

Finally, a further application of modified GLP-1RAs, in this case as an example of more selective tissue targeting, is the conjugation of vitamin B12 to exendin-4 that retains pancreatic actions but prevents central nervous system (CNS) penetration to reduce the side-effect of nausea [118].

Cyclic AMP binding proteins

Since the identification of cAMP as a second messenger elevated by GLP-1 [2], intense effort has gone into defining the downstream effectors that promote insulin biosynthesis, secretion and beta cell mass. Initially, cAMP was thought to signal exclusively through protein kinase A (PKA) to phosphorylate multiple targets, but identification of the Exchange Protein Activated by cAMP (Epac) led to its establishment as a second major pathway involved in cAMP signaling. A third class of cAMP binding proteins, the hyperpolarization and cyclic nucleotide gated (HCN) channels are functionally expressed in beta cells [119, 120], but their physiological significance remains unclear. The Popeye Domain Containing (POPDC) protein family constitutes a fourth class of cAMP-effector proteins [121] but their role in islets has not been examined. POPDC1/BVES and POPDC3 have been reported in the surface proteome and transcriptome of human islets [122, 123] and regulate several proteins [124] known to be important for islet function. These protein targets of POPDC1 include E-cadherin [125] that plays a role in human beta cell secretion [126] and apoptosis [127], VAMP3/cellubrevin that also plays a role in regulating secretion [127], and Rac1/Cdc42 [128] that play roles in secretion and cytoskeletal remodeling [129, 130]. At present, it is unknown which of the synthetic cAMP analogs (PKA- or Epac-selective) might activate POPDC proteins.

A novel indirect effector of cAMP signaling

An added layer of complexity to cAMP signaling is brought by the ability of the type 1 regulatory subunit of PKA (R1α) to directly bind and activate the phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 1 (P-Rex1) [131, 132]. This cAMP-dependent activation of P-Rex1 by R1α is in contrast to the inhibition of P-Rex1 by PKA-mediated phosphorylation [133]. It is also interesting to note that P-Rex1 is also activated by the βγ subunits of heterotrimeric G-proteins [134]. P-Rex1 expression in human islets is suggested in the human transcriptome [122] but its physiological role in islets has not been determined to date. It would, however, be predicted that P-Rex1 activation acting through Rac1 would promote insulin secretion in response to GLP-1R activation through effects on cytoskeletal

remodeling. Whether R1α might directly affect the activity of other proteins with the potential to regulate beta cell activity is currently unknown. An interesting role for P-Rex1 involves its interaction with the Gi-coupled sphingosine-1-phosphate (S1P) receptor-1 ($S1P_1$) [135]. The P-Rex1-$S1P_1$ interaction maintains the receptor at the cell surface through binding of the P-Rex1 PDZ-domain with the carboxy-terminal tail of the receptor, an effect that may also be applicable to other receptors [135]. S1P receptors are interesting as they play a protective role against apoptosis in beta cells in response to cytokines [136], in db/db mice [137] and in non-obese diabetic (NOD) mice [138], a model of type 1 diabetes. S1P signaling also plays a role in islet morphogenesis [139]. S1P might also play a role in regulating beta cell cAMP by acting as an agonist at the human GPR3, GPR6 and GPR12 receptors that activate AC (while also activating Gαi) and mobilize intracellular Ca^{2+} [140]. GPR3, 6 and 12 are observed in the surface proteome of human beta cells at low to moderate levels [123], but a functional role in beta cells has not been determined.

Signaling complexes regulated by cAMP and spatial localization of signals

Although this review focuses on cAMP signaling via the GLP-1R, it should be remembered that human islets express multiple GPCRs that can stimulate or inhibit cAMP production [141]. Several isoforms of adenylyl cyclase (AC) and phosphodiesterase (PDE) are also expressed in islets that can preferentially couple GPCR signaling, Ca^{2+} signals or metabolism to the regulation of cAMP levels [142]. These observations suggest the importance of spatial localization of the elements of cAMP production, a facet of islet biology that has not been extensively explored despite the demonstration over 20 years ago that A-kinase anchoring proteins (AKAPs) play a role in GLP-1 [143] and GCG [144] signaling in beta cells. There are many isoforms of AKAP that form GPCR-linked signaling complexes containing multiple proteins that can include AC, PKA, Epac and PDE isoforms [145]. These complexes allow very close spatial control of cAMP signals such that cAMP generated downstream of one type of GPCR does not enter the domain of another GPCR [146]. Although the composition of the macromolecular complexes regulating cAMP signaling in

islets is unclear, some relevant observations have been reported suggesting compartmentalization of signaling.

The type 5 isoform of AC (AC5) is essential for coupling glucose-metabolism to insulin secretion in human islets whereas it is not important for GLP-1-mediated effects [147]. In contrast, the type 8 AC (AC8) may be involved in both glucose- and GLP-1-regulated secretion [148-150], although AC8 expression in human islets is low or undetectable [122, 147] and requires further investigation. AC5 has been shown to be coupled to synaptic alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors by AKAP79 [151] and ionotropic AMPA receptors are expressed in islet beta cells where they stimulate insulin secretion [152, 153]. Whether AMPA receptors couple to AC5 in islets is unknown but their Ca^{2+} permeability [154] might be expected to inhibit AC5 and decrease cAMP levels. AKAP79 also inhibits Ca^{2+}-stimulated AC8 activity by recruiting PKA resulting in AC8 phosphorylation [155, 156]. From a pathophysiological perspective it is also interesting that, in HEK-293 cells, palmitoylation promotes AKAP79 association with lipid rafts and its regulation of AC8 [157] whereas, in neuronal synapses, CaMKII regulates depalmitoylation of AKAP79 [158]. These effects of CaMKII on depalmitoylation are independent of AKAP79 phosphorylation that inhibits its association with filamentous (F)-actin [158]. The depolymerization of F-actin was previously shown to disrupt AKAP79-cadherin interaction also resulting in the removal of AKAP from synapses [159]. Ca^{2+}-sensitive isoforms of AC, including AC5 and AC8, have been shown to localize to lipid rafts [160] where agonist-bound GLP-1R [107] and the protein components of store-operated Ca^{2+} entry (SOCE) also reside [161]. SOCE is reported to activate AC8 and stimulate cAMP production in MIN6 insulinoma cells [156] and may also inhibit AC5 in control liver cells [162]. Thus, it is possible to speculate that in obesity-related metabolic disorders, palmitoylation of AKAP79 will promote its localization to lipid rafts and inhibit both AC5 and AC8 with negative effects on insulin secretion. GLP-1 might reverse these effects by elevating intracellular Ca^{2+}, activating

CaMKII and depalmitoylating AKAP causing its removal and releasing the inhibition of AKAPs 5 and 8.

Activation of Epac2 by cAMP restores glucose-stimulated insulin secretion (GSIS) in type 2 diabetic human islets in a process that is, at least in part, mediated through restoring Cdc42-Pak1 activity resulting in actin remodeling [163]. This observation might also suggest that Epac2 activation, by changing F-actin levels, could release AKAP79 to enhance cAMP production in response to GLP-1. Disruption of lipid rafts has been shown to enhance both insulin [164] and GCG [165] secretion; whether changes in cAMP production as a result of AKAP79 distribution play a role in these effects is unknown. It is known that expression of the II-III intracellular loop of L-type Ca^{2+} channels, $Ca_V1.2$ and 1.3, causes their redistribution out of lipid rafts and inhibits both GLP-1-stimulated cAMP production and its potentiation of GSIS [166]. The mechanism underlying this effect on cAMP production is unknown, but the effect on secretion is likely to involve disruption of channel interaction with Munc13 [167]. The immediately releasable pool of insulin secretory granules is disrupted in T2DM and regulated secretion depends on the association of L-type Ca^{2+} channels with the granules mediated by Munc13 [167]. The type-II PKA regulatory subunits (RIIα and RIIβ) also associate with secretory granules and this association is regulated by unidentified AKAP(s) [168]. GLP-1-potentiated insulin secretion is substantially inhibited by disruption of RII-AKAP interaction [168]. Thus, there is a substantial body of evidence suggesting that spatial restriction of cAMP and Ca^{2+} signals are important for the regulation of insulin secretion.

Pathways and proteins involved in GLP-1/cAMP signaling in β cells

Aspects of cAMP signaling in the regulation of insulin and GCG secretion were recently reviewed [169] and more detailed general reviews of PKA [170] and Epac [171] signaling are also available. We will focus here on pathways known to be involved specifically in GLP-1 signaling, cAMP, PKA and Epac (Figure 2). These pathways result in changes in cell excitability, Ca^{2+} signals, trafficking, exocytosis, cell viability and cell-cell interactions.

However, given that a phosphoproteomics study on the synergy between glucose and GLP-1 to stimulate insulin secretion revealed over 5,000 proteins in beta cells that are phosphorylated [172], only a small subset will be discussed.

A: Insulin granule trafficking and exocytosis

Insulin granule trafficking and exocytosis was recently reviewed in detail [173] so we will address known effectors downstream of cAMP only. A role for both PKA and PKC in granule trafficking to enhance first and second phase insulin secretion has long been known [174], but little is known about the specific phosphoproteins involved. It is known that a pool of PKA anchored to secretory granules play an important role [168] and that PKA increases the sensitivity of exocytosis to Ca^{2+} [175] but the proteins targeted by PKA are unknown. An interesting observation is that PKA appears to have a permissive role that allows Epac activity to promote secretion [176] but the specific targets of PKA are again unknown. One protein that potentially plays a role in granule trafficking is syntabulin, a microtubule-associated protein that is phosphorylated downstream of Epac activation [177]. The kinase that phosphorylates syntabulin is unknown. A second protein phosphorylated by PKA is snapin that, in its phosphorylated state, interacts with the vesicular proteins SNAP-25, VAMP-2 (an interaction that is glucose-dependent), Epac2 and collectrin. Phosphorylation of snapin enhances GSIS and snapin phosphorylation is reduced in T2DM [178]. A third downstream effector of GLP-1 is synapses of amphids defective (SAD-A) kinase that has been shown to regulate Pak-1 and cytoskeletal remodeling [179], and to phosphorylate and inhibit Rho GDP-dissociation inhibitor (GDIα) to increase Rho activity and promote insulin secretion [180]. Finally, an interesting property of the delayed rectifier K^+ channel, $K_V2.1$, is that it can form aggregates and interact with syntaxin-1A to regulate exocytosis independently of its electrical activity [181], as discussed below. Whether the phosphorylation of $K_V2.1$ by PKA regulates this effect on exocytosis is unknown.

B: Changes in cell excitability

GLP-1 and cAMP signaling induce a modest increase in L-type Ca^{2+} channel activity in beta cells although cAMP has a more significant effect on exocytosis [182, 183]. In addition to enhanced Ca^{2+} channel activity, GLP-1 can inhibit repolarizing voltage-gated K^+ channels [184], previously shown to play a role in regulating GSIS [185]. The ATP-sensitive K^+ (K_{ATP}) channel that is the primary effector of glucose metabolism to depolarize beta cells [186] is also regulated by cAMP signaling. The ability of GLP-1 to act in synergy with glucose to inhibit K_{ATP} channels was shown to restore glucose-sensitivity to beta cells and excitability [187]. PKA was then shown to have complex actions on the molecular components of K_{ATP}, the pore-forming subunit Kir6.2 and the regulatory sulfonylurea receptor-1 (SUR1) expressed in beta cells. Thus, the phosphorylation of Kir6.2 was shown to increase channel activity whereas phosphorylation of SUR1 decreased channel open probability [188]. Later studies indicated that phosphorylation of SUR1 decreased the activating effect of ADP in the presence of low [ATP] [189]. A role for Epac2 in regulating K_{ATP} activity was identified where cAMP-dependent activation of Epac2 led to an increase in the ATP-sensitivity of the channel [190]. Subsequently, Epac-mediated activation of phospholipase Cε (PLCε) was shown to play a role in regulating GSIS [191]. PLCε is proposed to hydrolyze plasma membrane phosphatidylinositol 4,5 bisphosphate (PIP_2) that has an activating effect on K_{ATP} and this reduced activity increases the ATP- and sulfonylurea-sensitivity of the channel [192]. It was subsequently demonstrated that Epac activation does enhance the sulfonylurea sensitivity of K_{ATP} [193], which could in part explain the interaction of sulfonylureas and GLP-1RAs to induce hypoglycemia in T2DM patients [194]. In addition to enhanced sulfonylurea sensitivity of K_{ATP} at SUR1, it has also been suggested that some sulfonylureas can directly activate Epac2 through a cooperative interaction with cAMP [195].

The ability of K_{ATP} channel closure to cause depolarization depends on the presence of a depolarizing conductance. It is now thought that members of the transient receptor potential (TRP) family, that are known to be expressed in islets [196], are likely to be involved. GLP-1 was shown to activate TRPM2, a non-selective cation channel, through cAMP/Epac but not

through PKA signaling [197]. GLP-1 has also been shown to activate TRPM4 and TRPM5 through a PKC-dependent pathway [198]. This seems likely to occur downstream of Epac/PLCε, as described above, with diacylglycerol produced by PIP_2 hydrolysis activating PKC. TRPM2, 4 and 5 are all regulated by intracellular Ca^{2+} and thus may be activated by GLP-1-induced Ca^{2+} mobilization, as discussed below. The Ca^{2+} sensitivity of TRPM4 activation is increased by PKC-mediated phosphorylation [199].

A final mechanism by which excitability might be regulated is through volume-regulated anion channels (VRAC). These channels are depolarizing in beta cells and are activated not only by cell swelling as a consequence of elevated glucose, but also by cAMP and the sulfonylurea glyburide [200]. Which arm of the cAMP signaling pathway regulates these channels in beta cells is unknown but in other cells both positive and negative effects of Epac on channel activity have been reported along with a positive effect or no effect of PKA [201, 202]. It was recently shown that VRAC-forming LRRC8 plays a role in glucose sensing and the regulation of insulin secretion from beta cells [203]. Whether LRRC8 is regulated by GLP-1/cAMP is unknown but ERK1/2 appears to play a role in its phosphorylation [204] and ERK1/2 is activated by glucose and GLP-1 [205]. Thus, it is possible to speculate that LRRC8 might be regulated by interactions of glucose- and GLP-1 signaling to enhance excitability.

Two other types of anion channel, Ca^{2+}-activated anoctamin-1 (ANO1/TMEM16A) and cAMP-activated cystic fibrosis conductance regulator (CFTR), are reported to play a role in cAMP-amplified insulin secretion in human and mouse beta cells [206]. However, the expression of CFTR in human islets remains controversial and the development of cystic fibrosis-related diabetes may be primarily mediated by indirect effects rather than beta cell dysfunction [207, 208]. ANO1 and CFTR have been shown to form a signaling complex involving Epac1 and AC1 to associate closely with GPCRs in epithelial cells [209], but whether they form a complex in beta cells is unknown. Finally, another member of the

anoctamin family, ANO9, forms a cation channel that is activated by cAMP/PKA [210]. ANO9 is reported in the human islet transcriptome [122] but whether it is expressed in beta cells or its functional role in islets are unknown.

In addition to the depolarizing effects described above, GLP-1R activation can also inhibit repolarizing delayed-rectifier K^+ channels. This effect involves both cAMP/PKA and PI3 kinase/PKCzeta through GLP-1R-mediated transactivation of the EGF receptor (EGFR) [211]. This ability of the GLP-1R to transactivate the EGFR is a property shared by many GPCRs [212], but it remains to be determined which other beta cell receptors activate this pathway.

C: Intracellular Ca^{2+} release

Cholinergic stimulation activates muscarinic receptors M3 and M5 in human islets to stimulate insulin secretion and mobilize intracellular Ca^{2+} [213]. The source of acetylcholine in human islets is thought to be paracrine release from alpha cells [213], similar to the possible release of GLP-1 from alpha cells discussed earlier. This paracrine source of acetylcholine is likely to be important due to the sparse direct innervation in human islets [214]. The two major intracellular Ca^{2+} release channels in the endoplasmic reticulum (ER) are ryanodine receptors (RYR) and inositol trisphosphate receptors (IP_3R); both are present in beta cells and play a role in regulating secretion [215] and also cell death [216]. GLP-1-mediated mobilization of intracellular Ca^{2+} through a Ca^{2+}-induced Ca^{2+} release (CICR) process was demonstrated to occur through type 2 RYR [217] but it was unknown whether PKA or Epac was involved. IP_3R are gated by both IP_3 and Ca^{2+} and also exhibit CICR [218]. In beta cells, it was suggested that, downstream of GLP-1, PKA-mediated phosphorylation promotes CICR through IP_3R whereas Epac2 mediates effects on RYR [215]. More recent studies in mouse islets suggest that exendin-4, a GLP-1RA, causes Epac2-dependent Ca^{2+} mobilization through PLCε [219].

D: Cell viability

The main effect of GLP-1 signaling on beta cell viability in human islets comes from the inhibition of apoptosis. As discussed before, adult human beta cells, unlike rodent cells, have a limited capacity to proliferate. Many pathways have been identified to play a role in the prevention of apoptosis that will not be described in detail here. Among those pathways recently identified are SAD-A mediated phosphorylation of Bad (S155) [220], upregulation of BiP and JunB [221], increased Akt signaling and increased SIRT1 and α-Klotho [222], restoration of Pdx-1 expression [223], AMPK/mTOR/p70 S6K [224], and activation of the NOX2 pathway [225]. Both GLP-1 and GIP have also been shown to act through $K_V2.1$ to enhance beta cell survival [226]. Thus $K_V2.1$ acts as a mediator of GLP-1R signaling through effects on excitability, exocytosis and cell survival.

The inhibitory G protein, Gαz, reduces cAMP levels and plays a role in beta cell death in type 1 diabetes [227]. GLP-1 treatment synergistically interacts with Gαz deficiency to promote beta cell survival [228]. The association of Gαz with the plasma membrane is stabilized by Gβɤ subunits and by palmitoylation [229]. Thus, the deleterious effects of palmitate may be mediated in part through Gαz that has an inhibitory effect on GSIS [230] and could be overcome by GLP-1-stimulated cAMP production. Palmitoylation of Gαz also inhibits the GTPase activity of the regulators of G protein signaling (RGS) protein Gαz-GAP/RGS20 to prolong Gαz signaling [231] that is predicted to have a negative effect on beta cell function.

It should be noted that the effects of GLP-1R activation on cell viability, especially for human islets, have largely been demonstrated *in vitro*. Whether these effects contribute to the maintenance of beta cell mass *in vivo* during therapeutic use of receptor agonists remains unclear. It is also interesting that DPP-4 inhibitors have protective effects on human islets *in vitro* that are not blocked by exendin-(9-39), a GLP-1R antagonist, and occur as a result of NFkappaB inhibition [232]. The mechanism of this inhibition of NFkappaB by DPP-4

inhibitors remains uncertain and may be an effect of enzyme inhibition or a direct effect of the drug on unknown targets.

E: Cell-cell interactions

It was demonstrated that lipotoxicity disrupts cell-cell interactions in mouse and human islets and that this communication is enhanced by GLP-1. In human islets, it is suggested that GLP-1 recruits a network of beta cells that contributes to the functional beta cell mass [233]. Electrical coupling occurs through gap junctions formed by connexin-36 and coupling is decreased in pre-diabetic mice and also decreased by pro-inflammatory cytokines. These decreases in coupling can be reversed by cAMP elevation and the GLP-1RA exendin-4 can protect against the decoupling effect of cytokines [234]. The protective effect of cAMP is mediated by both PKA and Epac2, with PKA having a greater effect in human islets whereas Epac2 is more important in mouse islets [234]. Cytokine receptors can engage Gαz [235] but whether this plays a role in islet cell coupling is unknown.

Concluding Remarks

Retention of the incretin activity of GLP-1 and its receptor in T2DM has led to extensive investigation of this pathway to elucidate its ability to restore secretion from diabetic islets. Many new facets of receptor signaling have emerged in recent years that have yet to be fully explored but that raise the possibility of novel targets for therapeutic intervention. Some of these new aspects of GLP-1R signal transduction have been discussed here, including biased agonism, receptor recycling and novel effectors downstream of cAMP generation. Of particular importance will be the elucidation of subcellular compartmentalization of signals, englobing the spatiotemporal regulation of receptor activity as well as the compartmentalization of cAMP signaling into specific subcellular nanodomains. This is especially important now that receptor signaling in endocytic compartments has been reported, in addition to its traditional role within the plasma membrane. In summary, although our understanding of incretin pathways has increased dramatically, there still

remain many unknowns with the potential to explain, and possibly correct, the defects in secretion that occur in T2DM.

Figure Legends

Figure 1: Overview of GLP-1 actions. GLP-1 is secreted from intestinal L-cells [236] and regions of the brain [237] (reviewed in [238]). Under conditions of metabolic stress, GLP-1 may also be secreted by alpha cells within the islets of Langerhans where, along with GCG [46], it could act as a paracrine factor to regulate insulin secretion. GLP-1Rs in the brain play roles in regulating feeding and satiety and in glucoregulation [239], but can also induce nausea and other adverse gastrointestinal side-effects [240, 241]. GLP-1 inhibits gastric emptying [242] and, to a lesser extent, acid secretion. Several other extra-pancreatic tissues also respond to GLP-1 [243].

Figure 2: Glucose and GLP-1/cAMP signaling in beta cells. GLP-1 potentiates GSIS from beta cells through multiple pathways primarily mediated through cAMP and its downstream effectors PKA and Epac2. The soluble adenylyl cyclase (sAC or AC10) and AC5 are thought to play roles in glucose-stimulated cAMP production whereas AC8 is suggested to be important for GLP-1- and store-operated Ca^{2+} entry (SOCE)-stimulated cAMP production. Major effects of GLP-1 include the potentiation of glucose-induced changes in cell excitability, Ca^{2+} mobilization and exocytosis of dense-core insulin secretory granules (DCG). Not illustrated for clarity are effects on cell viability, gene expression, cytoskeletal remodeling and cell-cell connectivity, topics that are discussed briefly in the main text. Among the regulators of cell excitability are inhibition of K_{ATP} channels and voltage-gated potassium (K_v) channels, activation of the anion channels CFTR/ANO1 and the volume-regulated LRRC8, activation of non-selective cation channels of the TRP family and possibly ANO9, along with enhanced voltage-dependent Ca^{2+} channel (VDCC) activity. Ca^{2+} influx through both VDCCs and SOCE combined with Ca^{2+} mobilization through both

IP3R and RYR regulate exocytosis and also the Ca^{2+}-activated TRP and ANO1 channels, and Ca^{2+}-activated AC8. Cyclic AMP has important effects to enhance exocytosis through both PKA- and Epac2-mediated pathways. These pathways are discussed in more detail in the main text.

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Journal Pre-proof

Second GPCR target(s)	Additional therapeutic effect of second target(s)	References
GCGR	↑ weight loss via energy expenditure, hypocholesterolaemic effect	https://www.ncbi.nlm.nih.gov/pubmed/19602537 https://www.ncbi.nlm.nih.gov/pubmed/19597507 https://www.ncbi.nlm.nih.gov/pubmed/29945727
GIPR	Augmented incretin effect, ↑weight loss through unknown mechanism	https://www.ncbi.nlm.nih.gov/pubmed/30473097 https://www.ncbi.nlm.nih.gov/pubmed/30293770 https://www.ncbi.nlm.nih.gov/pubmed30578168
GLP-2R	Improved glycaemic control, potentially through improvements in gut integrity	https://www.ncbi.nlm.nih.gov/pubmed/29540315
NPY2R	↑ weight loss via appetite suppression	https://www.ncbi.nlm.nih.gov/pubmed/29491394 https://www.ncbi.nlm.nih.gov/pubmed/30471778 https://www.ncbi.nlm.nih.gov/pubmed/30622136
CCK1R	↑ weight loss via appetite suppression	https://www.ncbi.nlm.nih.gov/pubmed/25883113 https://www.ncbi.nlm.nih.gov/pubmed/29782892
CCK2R	Potential for increased beta cell mass	https://www.ncbi.nlm.nih.gov/pubmed/28928089
NTS1R	Increased insulin secretion, appetite reduction	https://www.ncbi.nlm.nih.gov/pubmed/30499633
GCGR & NPY2R		https://www.ncbi.nlm.nih.gov/pubmed/30622136

Table 1. Examples of secondary targets of bifunctional GLP-1R peptide hybrids

Journal Pre-proof

- GLP-1R signaling can be regulated by a number of biological processes.
- These include modulation of receptor trafficking and conformational dynamics.
- Dual/triple GLP-1RAs activating additional GPCRs have recently been developed.
- GLP-1R cAMP signaling is compartmentalized by ACs, PDEs and AKAPs.
- GLP-1R-induced cAMP activates multiple pathways to regulate beta cell function.

Journal Pre-proof

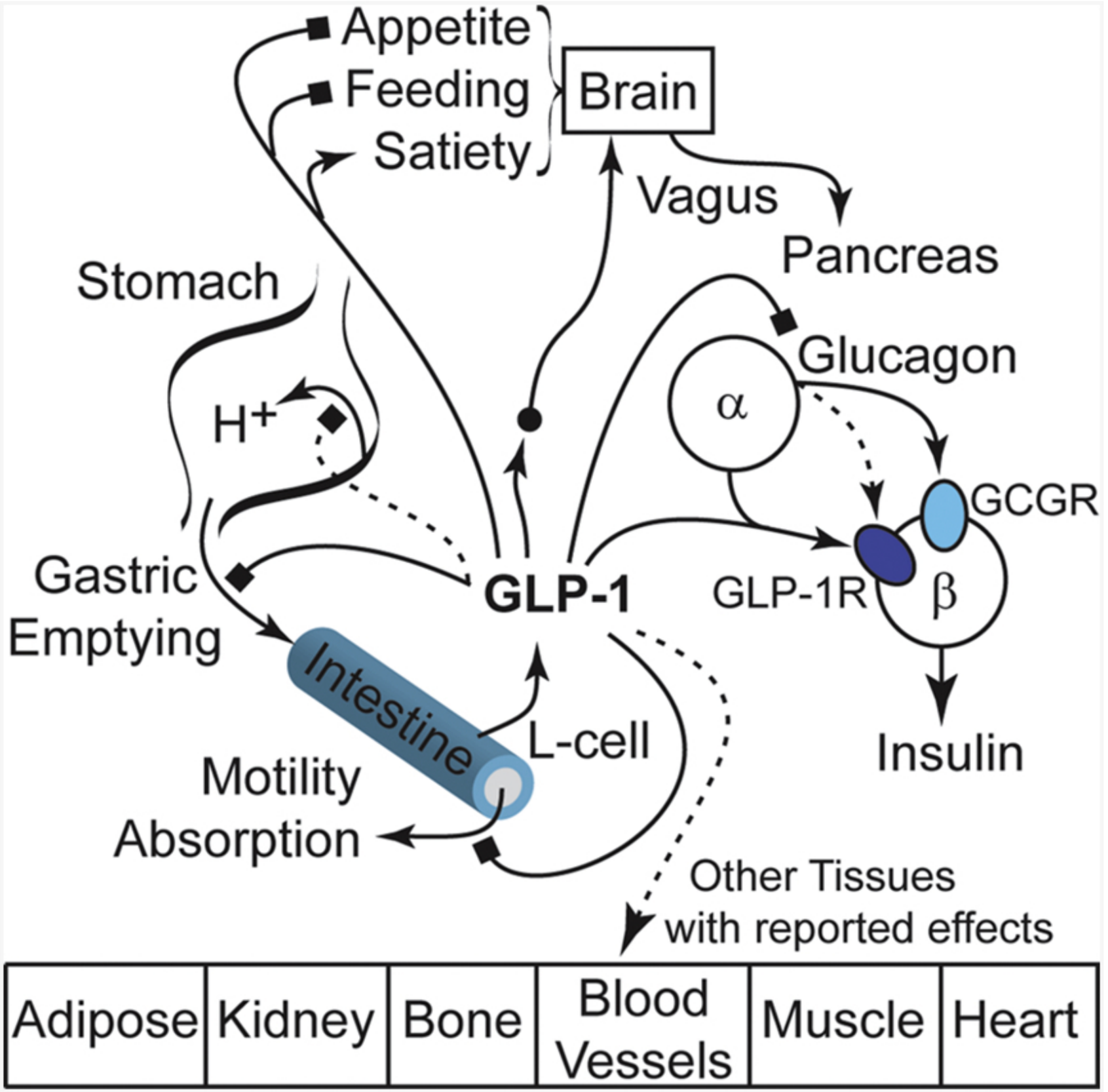

Appetite
Feeding
Satiety
Brain
Vagus
Pancreas
Stomach
Glucagon
α
H+
GCGR
Gastric
Emptying
GLP-1
GLP-1R
β
Intestine
L-cell
Insulin
Motility
Absorption
Other Tissues
with reported effects
Adipose
Kidney
Bone
Blood
Vessels
Muscle
Heart

Figure 1

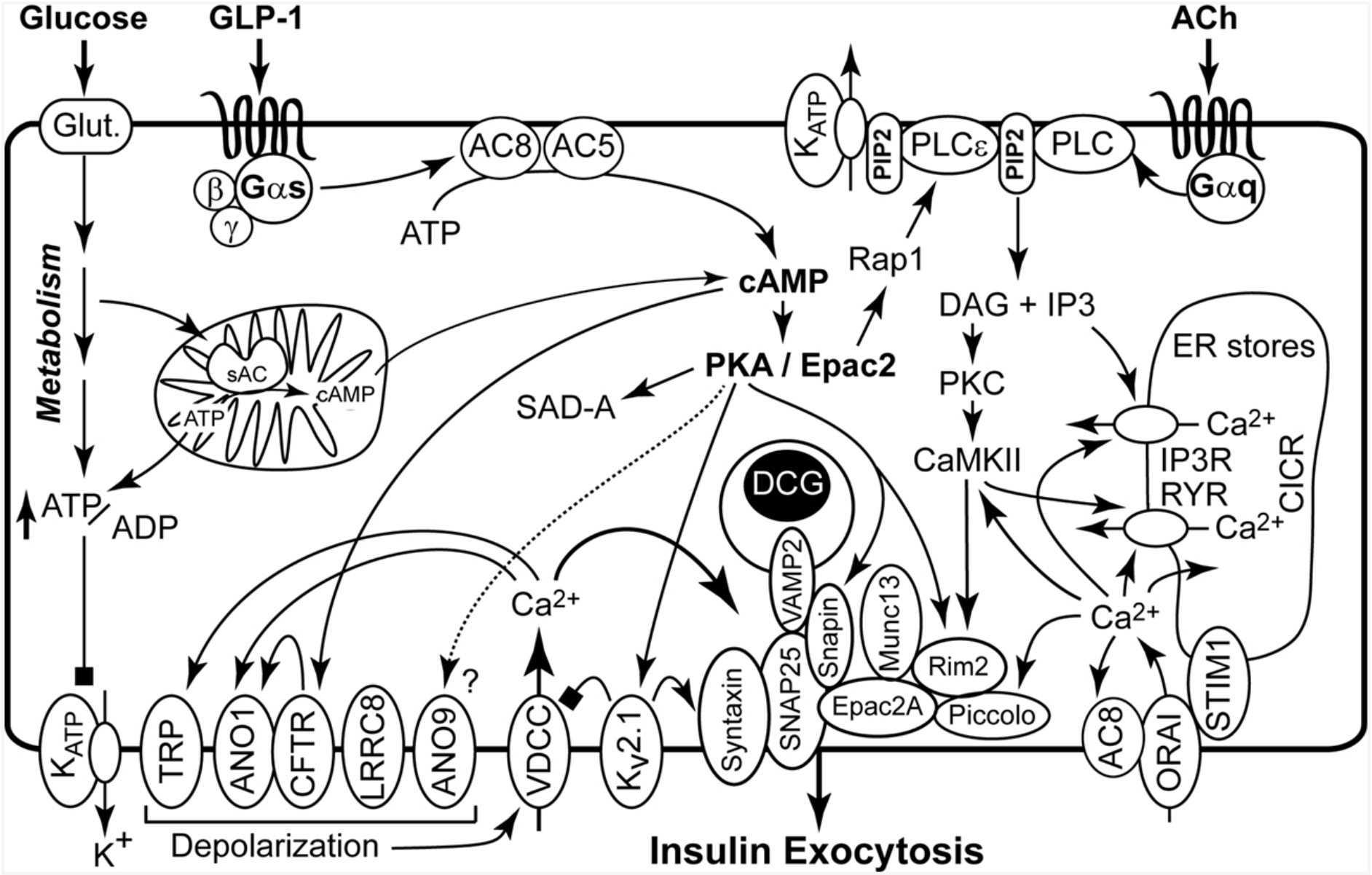

Glucose
GLP-1
ACh
Glut.
β
Gαs
γ
AC8
AC5
ATP
cAMP
Rap1
K_ATP
PIP2
PLCε
PIP2
PLC
Gαq
Metabolism
sAC
cAMP
ATP
PKA / Epac2
SAD-A
DAG + IP3
PKC
CaMKII
ER stores
Ca2+
IP3R
RYR
CICR
ATP
ADP
DCG
VAMP2
Snapin
Munc13
Rim2
Epac2A
Piccolo
Ca2+
Ca2+
Ca2+
K_ATP
TRP
ANO1
CFTR
LRRC8
ANO9
?
VDCC
Kv2.1
Syntaxin
SNAP25
AC8
ORAI
STIM1
K+
Depolarization
Insulin Exocytosis

Figure 2