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Revisiting the complexity of GLP-1 action-from sites of synthesis to receptor activation

Brent A. McLean[1,5], Chi Kin Wong[1,5], Jonathan E. Campbell[2], David J. Hodson[3], Stefan Trapp[4], Daniel J. Drucker[1,6]

From the Department of Medicine, Lunenfeld-Tanenbaum Research Institute, Mt. Sinai Hospital, University of Toronto[1], the Department of Medicine, Division of Endocrinology, Department of Pharmacology and Cancer Biology, Duke Molecular Physiology Institute, Duke University, 300 N Duke St, 27701 Durham, NC, USA[2], the Institute of Metabolism and Systems Research (IMSR), University of Birmingham, and Centre for Endocrinology, Diabetes and Metabolism, Birmingham Health Partners, Birmingham, UK [3], Centre for Cardiovascular and Metabolic Neuroscience, Department of Neuroscience, Physiology & Pharmacology, UCL, London, UK[4]

[5]BAM and CKW are co-first authors [6]DJD is the corresponding author and takes responsibility for the content of this manuscript

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Address correspondence to:

Dr. Daniel J. Drucker
LTRI, Mt. Sinai Hospital
600 University Ave Mailbox 39 TCP5-1004
Toronto Ontario M5G1X5
Canada

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Essential Points

The intestinal L cell network is the predominant contributor to circulating GLP-1

Brain-derived GLP-1 is widely distributed to multiple GLP-1Rs throughout the CNS

Plasticity of the endocrine pancreas enables the capacity for local GLP-1 production

Detection of the GLP-1R is challenging due to low level expression and technical validation of reagents

GLP-1 receptor agonists modify biology in cells and tissues that do not express the canonical GLP-1R

Abstract

Glucagon-like peptide-1 (GLP-1) is produced in gut endocrine cells and in the brain, and acts through hormonal and neural pathways to regulate islet function, satiety, and gut motility, supporting development of GLP-1 receptor (GLP-1R) agonists for the treatment of diabetes and obesity. Classic notions of GLP-1 acting as a meal-stimulated hormone from the distal gut are challenged by data supporting production of GLP-1 in the endocrine pancreas, and by the importance of brain-derived GLP-1 in the control of neural activity. Moreover, attribution of direct vs. indirect actions of GLP-1 is difficult, as many tissue and cellular targets of GLP-1 action do not exhibit robust or detectable GLP-1R expression. Furthermore, reliable detection of the GLP-1R is technically challenging, highly method-dependent, and subject to misinterpretation. Here we revisit the actions of GLP-1, scrutinizing key concepts supporting gut vs. extra-intestinal GLP-1 synthesis and secretion. We discuss new insights refining cellular localization of GLP-1R expression and integrate recent data to refine our understanding of how and where GLP-1 acts to control inflammation, cardiovascular function, islet hormone secretion, gastric emptying, appetite, and body weight. These findings update our knowledge of cell types and mechanisms linking endogenous vs. pharmacological GLP-1 action to activation of the canonical GLP-1R, and the control of metabolic activity in multiple organs.

Introduction

The gut endocrine system contains dozens of specialized plurihormonal endocrine cells whose peptide hormone products regulate a wide range of biological functions, ranging from control of ingestive behavior to the digestion, absorption, and assimilation of nutrients (1). Among the best studied of the gut hormones is glucagon-like peptide-1 (GLP-1), a product of the proglucagon gene (*GCG*). The cloning of the proglucagon cDNAs encoding GLP-1 within the proglucagon (*GCG*) gene in the early 1980s (2, 3), enabled the study of the physiology of the glucagon-like peptides. Originally described as an incretin hormone that potentiates meal-stimulated insulin secretion, several decades of research have broadened the actions of GLP-1 beyond the endocrine pancreas to encompass activity within the nervous system, gut, kidney, pancreas, heart, and immune system (4-6). Moreover, the clinical development of GLP-1R agonists (GLP-1RA) for the treatment of type 2 diabetes (T2D) and obesity has heightened the interest in how both physiological and pharmacological levels of GLP-1 act on key tissues relevant to the pathophysiology of diabetes and its complications.

A major advance in understanding GLP-1 action stemmed from the cloning and characterization of the GLP-1 receptor (GLP-1R) (7). *GLP1R* mRNA transcripts were found to be widely distributed in peripheral tissues and the central nervous system (CNS), providing a logical link between the demonstrated actions of GLP-1 and the expression of the canonical GLP-1R in cellular targets of GLP-1 action (8-10). Nevertheless, the precise cellular localization of GLP-1R has proved to be challenging, reflecting low levels of mRNA expression, and multiple technical challenges, including the inadvertent application of incompletely characterized non-specific antisera for detection of GLP-1R protein expression (11).

The concept of GLP-1 as a gut-derived hormone acting on peripheral organs, has been refined through enhanced understanding of the importance of pancreatic- and brain-derived GLP-1. GLP-1 circulates at very low (picomolar) levels, is immediately inactivated by local gut and systemic expression of dipeptidyl peptidase-4 (DPP-4) (12, 13), and is rapidly cleared from the circulation by the kidney. Contemporaneously, evidence supporting the pancreatic production of GLP-1 has fostered the competing notion that the incretin activity of GLP-1 reflects local GLP-1 synthesis in α-cells, coupled with paracrine actions on adjacent GLP-1R+ β-cells (6). Moreover, notwithstanding the reduction of food intake and weight loss observed with pharmacological administration of GLP-1R agonists (GLP-1RA), inactivation of gut GLP-1 production does not perturb control of food intake or body weight (14), consistent with considerable evidence implicating local brainstem-derived GLP-1, and not gut-derived GLP-1, as a key contributor to GLP-1R-dependent signals regulating appetite and body weight.

Similarly enigmatic is the challenge of understanding the actions of GLP-1 and degradation-resistant GLP-1RA on organs and cell types that do not express the GLP-1R. For example, GLP-1RA reduce the rates and extent of myocardial infarction in humans and in preclinical studies (15-17), yet it remains challenging to detect GLP-1R protein expression in the left ventricle of the heart, the most common site of infarction (18). Similarly, GLP-1RA reduce hepatic fat accumulation and are under clinical investigation for the treatment of non-alcoholic steatosis, yet hepatocytes do not appear to express a functional canonical GLP-1R (19-21). Moreover, GLP-1RA rapidly reduce the levels of postprandial triglycerides in animals and humans, yet enterocytes do not exhibit GLP-1R expression or activity. These and related examples highlight how multiple pharmacological actions of GLP-1 and GLP-1RA are highly

conserved across species, yet difficult to explain mechanistically based on the known expression patterns of the GLP-1R.

Several technological advances have enabled re-examination of these questions, refining our understanding of where and how GLP-1 is synthesized and acts to originate GLP-1R-dependent signals in multiple organs. Similarly, new tools have evolved for more sensitive and reliable detection of GLP-1R expression. These include single cell RNA sequencing (scRNA-seq, reporter mice illuminating cellular sites of *Glp1r* transcriptional activity, more sensitive and specific validated GLP-1R antisera, advanced microscopy, and new chemical probes for enhanced detection of GLP-1R binding sites at single cell resolution.

Here we re-evaluate existing concepts of GLP-1 action, highlighting insights into established and emerging concepts illuminated by recent progress in the evaluation of GLP-1 production and in the expression and activity of the canonical GLP-1R. The sites of GLP-1 synthesis within the gastrointestinal tract, the brain, and the pancreas are described, and linked where possible, to actions of GLP-1 within these organs, which are transduced by the canonical GLP-1R. We focus on discussing established and emerging concepts of how circulating GLP-1 produces multiple actions, both directly, and indirectly, on multiple organs and cell types with variable GLP-1R expression. Collectively, these findings update established and emerging concepts of GLP-1 action, and highlight mechanistic controversies surrounding GLP-1 action on cell types that do not express the GLP-1R.

The Pancreas

GLP-1 production, GLP-1R expression and GLP-1R signaling in the pancreas

Is pancreas-derived GLP-1 relevant for islet function?

An ongoing debate centres on whether proglucagon processing in α-cells is capable of producing meaningful amounts of bioactive GLP-1 to influence β-cell function and glucose tolerance. Early reports demonstrated increased α-cell expression of *Pcsk1*, the prohormone processing enzyme required for GLP-1 production, in response to pancreatic injury or induction of experimental pancreatic inflammation (22-26), raising the hypothesis that α-cells could adapt to metabolic stress by increasing GLP-1 production. Active GLP-1 was detected, albeit at low levels relative to corresponding levels of glucagon in the same extracts, from both mouse and human pancreas (14). However, induction of metabolic stress in high fat diet (HFD)-fed mice did not alter the levels of GLP-1 in the pancreas (14). Genetic deletion of *Pcsk1* to eliminate prohormone convertase 1 (PC1) reduced islet content and secretion of GLP-1 by ~40%, leading to modest impairment of glucose intolerance in obese mice (25), reinforcing the key role of PC1 in α-cell GLP-1 production. However, analyses using the mouse perfused pancreas and a specific assay to detect active, fully processed GLP-1 reported mostly undetectable levels of active GLP-1 in the perfusate, arguing that α-cell GLP-1 production in the normal murine pancreas is negligible (27). On the other hand, the GLP-1R antagonist exendin(9-39) (Ex9) did not impair glycemic excursion in *Gcg*$^{-/-}$ mice with preserved intestinal GLP-1 production, yet selective rescue of pancreatic *Gcg* expression in *Gcg*$^{-/-}$ mice implicated pancreatic GLP-1 in the control of glucose homeostasis in this mouse model (28). Complicating this interpretation are findings that glucagon, produced at much higher levels in the islets, is also capable of stimulating insulin secretion and controlling glucose homeostasis through the GLP-1R (27, 29-31). Consequently, paracrine interactions between α- and β-cells mediated by *GCG* peptides

and the GLP-1R could be completely accounted for by glucagon secretion. Interestingly, while the levels of glucagon secreted from isolated mouse islets are ~300-fold greater than GLP-1, the potency of GLP-1 for insulin secretion is ~300-fold more than glucagon (31). Finally, if α-cells produce active GLP-1, this source does not contribute to the circulating concentrations in the plasma as mice with intestine-specific inactivation of the *Gcg* gene exhibit almost undetectable levels of active circulating GLP-1 (14). **Figure 1A** summarises paracrine GLP1 signaling in the islet.

GLP1R/Glp1r **mRNA expression in different islet cell types**

Prior to the development of validated antibodies for the GLP-1R, the expression patterns of *Glp1r/GLP1R* in islet cells were derived from analysis of ligand binding and RNA levels. The GLP-1R cDNAs were originally cloned from rat (7) and human (32) pancreas, and later shown to be expressed in the mouse pancreas by RT-PCR (10). Currently, islets/pancreas samples are often used as a positive control when exploring *Glp1r/GLP1R* expression in other tissues (18). However, it is continuously debated which endocrine cell population within the islet express the GLP-1R. Multiple strategies have been used to identify *Glp1r/GLP1R*+ cell populations, including transcriptional analysis of sorted islet populations. Using genetic reporter lines to enrich for populations of α-, β-, and δ-cells, *Glp1r* was shown to be robustly expressed in mouse β-cells, moderately expressed in δ-cells, and expressed at low to undetectable levels in α-cells (33). These data align with a study employing bulk RNAseq to analyze enriched cell populations obtained from reporter mice, showing *Glp1r* to be highly expressed in β-cells, less so in δ-cells, but absent in α-cells (34).

An alternative approach to identifying islet *Glp1r* localization utilized mice expressing Cre recombinase under control of the *Glp1r* promoter. Using *Glp1r-Cre* mice crossed with a fluorescent reporter line, cells are indelibly marked in response to *Glp1r* transcription (35). Such approaches are of high fidelity, since only a few molecules of Cre are required to excise the stop cassette and drive reporter protein expression. A downside of this approach is the potential disconnect between transcription and translation, although this is less likely in light of newer data (see below). Moreover, relative levels of GLP-1R cannot be determined between and within cell types, since reporter expression is generally driven using a Rosa26 promoter.

The first study to utilize this approach to investigate *Glp1r* promoter activity in islets reported strong activity in both β- and δ-cells, although *Glp1r* levels were ~10-fold higher in the β-cell population (35). Subsequent analysis of α-cell populations with double reporter mice (*Glp1r-Cre:ROSA26-tdRFP:GLU-Venus*) produced an estimate that 9.5% of α-cells are *Glp1r*+. Interestingly, the fraction of *Glp1r*+ α-cells also contained significantly more *Ins1* expression compared to *Glp1r*- α-cells. Could these represent a sub-population of unique α-cells that are GLP-1 responsive? Do they arise from de-differentiated β-cells, explaining the presence of both *Glp1r* and *Ins* transcripts? Or do these results arise from contamination of a non-α-cell population? The importance of this subpopulation of α-cells remains unclear. A second study examined *Glp1r* activity in islet cell populations using an independent *Glp1r-Cre* mouse crossed with the mTmG reporter mouse line (36). The expression of *Ins* and *Sst* were found in the *Glp1r*+ population (GFP+), while *Gcg* expression was exclusive to the *Glp1r*- population (TdTomato+). This data set indicates little to no *Glp1r* expression in α-cells, conflicting with previous reports, but this approach cannot rule out a small population of α-cells being *Glp1r* positive.

scRNA-seq has been utilized for interrogation of the *Glp1r* expressing cell population within islets, and to assess for potential heterogeneity in expression within a particular cell population. For instance, in samples from human islets, *GLP1R mRNA* was robustly detectable in β- and δ-cell populations, with very few *GLP1R*+ α-cells (37), largely aligning with the results of cell population analysis from transgenic mice described above (**Figure 2**). However, it was interesting to note the considerable range in expression levels between individual β-cells (and δ-cells), particularly since a significant portion of individual cells in either population were reported to be *GLP1R*-. Heterogeneity in β-cell *GLP1R* expression was common amongst all human and mouse scRNA-seq data analyzed (38), indicating this outcome is consistent despite the varied platforms used to obtain these data. It is exciting to imagine heterogeneity in the relative expression of *GLP1R/Glp1r* within specific cell populations, as this could reconcile the reports that only a fraction of α-cells are GLP-1R+ and potentially contribute to the ongoing discussion of β-cell heterogeneity (38, 39). However, the varying levels of GLP-1R transcripts in different cells was subsequently proposed to reflect technological limitations associated with scRNA-seq, due to the significant drop out of low-expressing genes during the process of generating the cDNA library (39).

GLP-1R protein expression and localization in the rodent islet

Multiple approaches have localized GLP-1R in pancreatic islets. Early studies using immunohistochemistry (IHC) to examine rat pancreas showed immunoreactivity for GLP-1R in the majority of β-cells and δ-cells, with 1 in 5 α-cells also expressing the receptor, in line with single cell RT-PCR findings (40). Later studies using the same rat antibody showed that GLP-

1R was predominantly expressed in the β-cell compartment, with no detectable GLP-1R protein or mRNA in α-cells and δ-cells (41, 42). This was further reflected in studies by an independent group using two commercial antibodies (42) although one of the reagents was subsequently shown to lack specificity for the GLP-1R (43). All of these early studies detected GLP-1R in the β-cell membrane as well as in the cytoplasm. While this could reflect the imaging technique used, cytoplasmic localization could also reflect non-specific signal, since GLP-1R should be present predominantly at the plasma membrane in its unstimulated state (**Figure 1B**). While a validated monoclonal antibody (Mab 7F38) has been reported which displays a clean membrane signal (44), it has not yet been used to extensively re-examine GLP-1R cellular localization in mouse islets. Recent studies using a novel antibody raised against the GLP-1R *N*-terminal region, and validated using a new floxed GLP-1R-null reporter mouse line, detected GLP-1R throughout the β-cell and α-cell cytoplasm (45). The reason for this discrepancy in the expression of the α-cell GLP-1R across studies is unclear, but might reflect the use of antibodies raised against different GLP-1R epitopes (e.g. *N*-terminus or the wider extracellular domain).

A second approach to detect GLP-1R in islets relies upon orthosteric agonists or antagonists conjugated to various fluorophores (Cy3, Cy5, TMR, SiR) (46-48). Inherent advantages of this approach include visualization of GLP-1R capable of ligand binding and activation and live tissue imaging, which reduces background signal introduced by fixation. However, because peptides and some fluorophores are cell impermeable, GLP-1R already within the cell will not be readily detectable. Studies using fluorescent agonists reported similar findings to those with monoclonal antibody: GLP-1R expression largely confined to the β-cell compartment, with expression in a small subpopulation of α-cells (~ 1%) (48). Along these lines, well characterized

fluorescent antagonists revealed predominant GLP-1R expression in β-cells (~85%), with slightly higher expression in α-cells (~ 5%) than shown with fluorescent agonist or antibody (49) but in agreement with *Glp1r-Cre:ROSA26-tdRFP:GLU-Venus* double reporter mouse approaches (35). Notably, for all fluorescent-ligand localization approaches, staining overlapped with data obtained using IHC with antibodies validated in *Glp1r*$^{-/-}$ mouse tissue (48, 49).

GLP-1R protein expression and localization in the primate and human islet

Most of the studies investigating GLP-1R distribution in primate and human islets have used IHC. Using a validated monoclonal antibody (Mab 3F52), GLP-1R co-localized with insulin-positive cells in the monkey islet (44). Conversely, less co-localization was detected in glucagon-positive cells, with the observed overlap attributed to cytoplasmic extensions from β-cells (44). However, given the axial resolutions needed to accurately separate cell membranes, further studies will be required to exclude GLP-1R expression in monkey α-cells.

Early studies looking at human islets showed strong co-localization of GLP-1R with β-cells, with no staining seen in α-cells (41). Similarly, studies using a commercial antibody confirmed these results, showing GLP-1R immunopositivity in 0.25% of α-cells (50). It is worth noting however that the antibodies used in both these studies gave rise to predominantly cytoplasmic staining, at odds with the known localization of non-stimulated GLP-1R. Studies using a validated antibody with membrane-localized staining again showed GLP-1R expression largely confined to β-cells, but staining was not performed for glucagon, making quantification impossible (44). Consistent with these observations, Waser and colleagues utilized the Mab 3F52 antibody and detected membrane-localized GLP-1R-immunopositivity in β-cells and δ-cells, but not in α cells

290 from human pancreata resected from individuals with insulinomas, chronic pancreatitis, or
291 pancreatic cancer (51). Most recently, studies using the same antibody combined with confocal
292 imaging showed that GLP-1R is expressed in most β cells and very few α-cells (52). Of interest,
293 GLP-1R was shown to be moderately expressed in somatostatin+ (i.e. δ-cells) (52). Thus,
294 based upon IHC, GLP-1R expression patterns in human reflect those seen in the mouse, but
295 with some immunoreactivity also detected in δ-cells (the majority of mouse studies did not
296 quantify somatostatin+ cells).
297
298 ***Tying together GLP-1R transcript and protein levels in the same cell***
299 While the measurement of *Glp1r* mRNA transcripts is not burdened with the limitations present
300 when assessing protein levels based on current approaches, extending *Glp1r* transcript
301 abundance to protein is a necessary step to identify cells that permit GLP-1 activity. However,
302 there appears to be significant discordance between detection of gene and protein expression
303 of the GLP-1R in mouse islets (36). These observations stemmed from sequential efforts to first
304 validate a novel GLP-1R antibody (53) in *Glp1r*$^{-/-}$ mice to demonstrate specificity for the GLP-
305 1R in dispersed islets cells during flow cytometry. Next, this antibody was applied to *Glp1r-*
306 *Cre:mTmG* reporter mice to compare *Glp1r* promoter activity with GLP-1R protein levels. The
307 antibody failed to stain any TdTomato+ cells (*Glp1r*-), enhancing the validity of the two reagents
308 (36). This population of *Glp1r*- cells were enriched for *Gcg*, marking this population as α-cells.
309 Remarkably, two populations emerged with respect to GLP-1R staining when examining the
310 GFP+ cells (*Glp1r*+). The population of GFP+ cells that stained positive with the antibody were
311 enriched for *Ins2* expression, while the population of cells that stained significantly lower with
312 the antibody were enriched for *Sst* (36). This suggests that β-cells have robust GLP-1R protein
313 levels, and that δ-cells have much less GLP-1R. These findings were corroborated in

experiments in WT islets, where the GLP-1R antibody robustly stained β-cells, but failed to detect protein expression in either α- or δ-cells. The potential discrepancy between RNA and protein expression in δ-cells warrants further investigation to eliminate the potential contribution of technical limitations, especially given the relative paucity of this cell type. Finally, the majority of β-cells express the GLP-1R and this percentage was not changed by various stressors reported to modulate *Glp1r* levels, indicating little plasticity in expression of the β-cell GLP-1R.

Consensus view of islet GLP-1R expression

Given the number of studies, what is the consensus view of GLP-1R expression in the islet? It is clear that β-cells abundantly express GLP-1R (80-95%), whilst the receptor is absent or present in a very small proportion in α-cells (0-20%) (35, 36, 40, 41, 48, 49). δ-cells are less well-characterized and although some analyses reveal expression of the *Glp1r* transcript in these cells (33, 34), analyses at the protein level are less firm showing little to no GLP-1R expression (36, 41, 46, 49). These figures should however be interpreted with some limitations. Quantification based upon IHC is notoriously challenging. Indeed, it can be difficult to assign GLP-1R expression to adjacent cells whose membranes overlap (as is often the case for α-cells, β-cells and δ-cells) (49). Non-specific background signal in cells expressing low levels of GLP-1R is problematic, and differences in sensitivity/settings between different microscopes means that results are difficult if not impossible to calibrate across studies (compared with RNA-seq and FACS which are more standardized). On the other hand, visualizing GLP-1R expression in the intact tissue avoids artefacts introduced by cell dissociation as well as removal of cells from the tissue setting. Moreover, utilizing FACS to isolate endocrine cell populations based on primary hormone expression can mask heterogeneous *Glp1r* expression in

subpopulations of cells. For instance, if less than 10% of α-cells are *Glp1r*+, the remaining 90+% of α-cells in the binned sample may dilute this signal to yield the interpretation that α-cells are *Glp1r*-. Thus, combining multiple approaches to interrogate GLP-1R expression remains necessary to account for the limitations of a single approach. Table 1 summarizes GLP-1R transcript and protein levels based on the studies outlined above.

Where and how does GLP-1 act in the islet?

β-cells: The molecular pharmacology of GLP-1R signalling and trafficking has been extensively studied in heterologous expression systems and is reviewed in detail elsewhere (54, 55). Here we briefly cover facets of GLP-1R signalling pertinent to insulin release from primary β-cells where the investigation of the sites and mechanisms of GLP-1R signalling remain less well characterized. Activation of the GLP-1R strongly induces adenylate cyclase and cyclic adenosine 3′,5′-monophosphate (cAMP) accumulation following ligand activation, with nanomolar concentrations of GLP-1/Exendin-4 (Ex4) acting through cAMP and exchange protein directly activated by cAMP (EPAC) pathways in β-cells (56). cAMP acts as a potent stimulus of insulin secretory granule exocytosis by increasing voltage-dependent Ca^{2+} channel activity, Ca^{2+} influx, as well as directly sensitizing vesicles themselves for release (57, 58). While cAMP constitutes the major mediator of GLP-1R signalling, contributions from β-arrestin are more debated. On one hand, both β-arrestin 1 (59) and β-arrestin 2 (60, 61) have been shown to be dispensable for acute stimulation of insulin secretion by GLP-1/GLP-1RA. However, other studies have shown that β-cell knockout of *Arrb1* enhances insulin secretion in response to GLP-1, suggesting β-arrestin 1 limits GLP-1R signalling in β-cells (62).

The differing concentrations of GLP-1 used in these experiments could reconcile the discrepant results in the β-arrestin 1 knockout models, as high concentrations of GLP-1 (100 nM) (59) may elicit different signaling pathways compared to GLP-1R activation with lower concentrations (0.3 nM) (62). Moreover, the absence of a phenotype in the single β-arrestin knockout models (59-61) could equally reflect the presence of redundancy in the GLP-1R signalling network and as such studies in double β-arrestin 1/2 knockout mice are needed. The major GLP-1R signaling pathways in rodents and human β-cells are shown in **Figure 1C.**

Activation of GLP-1R by picomolar concentrations of GLP-1 has been demonstrated in mouse and human β-cells (63, 64). Rather than engaging cAMP synthesis, low concentrations of GLP-1 instead activate a phospholipase C (PLC)-protein kinase C (PKC)-Transient Receptor Potential Cation Channel Subfamily M Member (TRPM)4/5-signalling cascade through the Gqα subunit, stimulating insulin secretion (63). Low concentrations of GLP-1 also to increase Ca^{2+} fluxes in human β-cells, which fits with a role for Inositol 1,4,5-trisphosphate 3(IP3)-dependent mobilization of endoplasmic reticulum (ER) Ca^{2+} stores (IP3 being downstream of PKC) (63, 64). Most recently, GLP-1R has been shown to signal predominantly via Gqα in mouse and human β-cells subject to chronic depolarizing stimuli (K_{ATP} channel mutations, hyperglycemia) (65). Since several of these experiments used chemical inhibitors with known off-target effects (e.g. on store-operated Ca^{2+} entry, phosphodiesterases), it will be important to examine whether similar phenotypes exist in β-cells specifically deleted or knocked down for Gqα or Gsα. Lastly, GLP-1 also engages a network of β-cells in human islets, leading to coordinated Ca^{2+} rises (64). Such signalling is dependent on intercellular gap junction communication, which Ex4 has been shown to facilitate in a cAMP-protein kinase A (PKA)-EPAC-dependent manner (66).

α-cells: The hypothesis that GLP-1 directly affects α-cell function remains controversial. Despite evidence supporting GLP-1R expression in only a small subpopulation of α-cells, studies have shown that GLP-1 influences α-cell signalling and glucagon secretion by inhibiting N and P/Q voltage-dependent Ca^{2+} channels (42, 45, 50). α-cells could plausibly express low levels of GLP1-R, which would be difficult to detect using immunostaining, yet sufficient to evoke a response in a highly-amplified signalling cascade. However, low abundance *Glp1r* mRNA transcripts should still lead to recombination in the various GLP-1R-reporter mice discussed above, where somewhere between 0% (36) and 10% (35) of α-cells were labelled with reporter. Moreover, the most recent fluorophore antagonist approaches have single-molecule resolution and detected GLP-1R in only 1-5% of α-cells, with either complete absence or strong signal, suggesting that graded expression in the population is unlikely (49). Another explanation for the actions of GLP-1 on α-cells could be through GLP-1 degradation products. Several reports suggest that GLP-1(7-36) and GLP-1(9-36) are glucagonostatic in wild-type and *Glp1r*$^{-/-}$ islets (67). Effects of GLP-1(7-36) but not GLP-1(9-36) were abolished by pre-treatment with a DPP-4 inhibitor, suggesting that GLP-1(7-36) is pro-peptide for GLP-1(9-36) in this setting (67). Notably, GLP-1(9-36) was found to signal at the human glucagon receptor with an EC_{50} = 30 nM. While such concentrations are pharmacological, GLP-1(9-36) is the major circulating form of GLP-1 and DPP4 is present in islets, which could plausibly generate high local levels of cleavage products. These findings are however difficult to reconcile with previous studies showing that the GLP-1R-specific antagonist Ex9 (27) blocks the effects of GLP-1 on α-cell electrical activity and glucagon secretion (42). Although interesting, these data are unlikely to be relevant for degradation-resistant GLP-1RA and DPP-4 inhibitors, which suppress glucagon secretion without generating GLP-1(9-36) through mechanisms requiring the known GLP-1R.

δ-cells: Relatively few reports demonstrate direct actions of GLP-1 on somatostatin secretion. Studies using the perfused mouse pancreas preparation showed that GLP-1 lowers glucagon secretion in a paracrine manner via an increase in somatostatin output (which negatively regulates α-cell function) (68). Notably, the action of GLP-1 to inhibit glucagon secretion was prevented by blocking somatostatin receptor 2 (SSTR2), which is expressed on α-cells and β-cells (69). Similar results have been reported using the perfused rat pancreas (70). Such paracrine regulation of glucagon secretion by GLP-1 likely requires an intact microvasculature, since the SSTR antagonist CYN154806 did not block the glucagonostatic effects of GLP-1 in isolated mouse islets (42). It remains unclear how GLP1 increases somatostatin secretion in mouse δ-cells given the low levels of GLP-1R protein detected following FACS to isolate islet cells (see above). The expression and functional importance of the GLP-1R in δ-cells in the intact pancreas, and the consequences arising from conditional deletion of the GLP-1R in the δ-cell compartment requires further analysis.

GLP-1R expression and function in the exocrine pancreas

Interest in the roles of GLP-1R expression and activity in pancreatic acinar cells was heightened by the hypothesis that the clinical use of GLP-1RA might be linked to the development of pancreatitis or pancreatic cancer (11). Indeed, GLP-1RA such as exenatide or liraglutide increase pancreatic mass and protein synthesis independent of changes in cell proliferation in mice (71). Moreover, native GLP-1 directly increases pancreatic enzyme secretion from and cAMP formation in mouse pancreatic acinar cells (72), actions requiring the canonical GLP-1R. The *Glp1r* mRNA transcript is detectable in RNA isolated from mouse pancreatic acini (72), and weak variable GLP-1R-immunopositivity was detected in in pancreatic acinar cells, but not in ductal cells, with the Mab 3F52 antibody in sections from pancreata obtained from monkeys

with or without diabetes (44). Similar patterns of acinar cell GLP-1R-immunopositivity were detected in pancreatic specimens obtained at surgery from 10 non-diabetic individuals, as well as in 12 pancreatic specimens from individuals with T2D. In situ ligand binding detected GLP-1 binding sites in the pancreatic acinar cells, with radiolabelled ligand displaced in the presence of excess unlabelled GLP-1 (44). Waser and colleagues examined pancreatic GLP-R expression in the human pancreas by IHC using the Mab 3F52 antibody, as well as by in situ ligand binding autoradiography using ^{125}I- GLP1(7–36)-amide (51). A combination of both diffuse and heterogeneous weak GLP-1R expression was observed in some but not all acini from pancreatic samples resected from insulinoma tumors, chronic pancreatitis and a few cases of pancreatic cancer. No immunoreactive GLP-1R expression was detected in pancreatic ducts (51). Similar patterns of acinar cell GLP-1R localization were reported in the mouse pancreas, using the ^{125}I-labeled GLP-1R antagonist Ex9 to detect in situ binding, as well as the Mab 7F38 antibody to localize GLP-1R immunopositivity in the exocrine pancreas of WT but not *Glp1r*$^{-/-}$ mice (73). Hence, the available data supports low level GLP-1R expression in the pancreatic acinar but not ductal cells in mice and humans.

Concluding remarks for GLP-1R expression and function in the pancreas

Actions mediated by GLP-1 in the islet are shown in **Figure 1D.** The major source of GLP-1 that binds GLP-1R in the healthy islet is still unresolved, although GLP-1 produced in α-cells may play a relatively more important role in the injured pancreas. Insulinotropic effects of GLP-1 and GLP1-RA are mediated by GLP-1Rs expressed predominantly (if not completely) in the β-cell compartment in both mice and human islets, assessed using validated antibodies, probes and reporter mice across a range of techniques. Direct effects of GLP-1 on α-cell function and glucagon secretion remain debated and are unlikely to be mediated by the GLP-1R. Instead,

degradation of GLP-1 by DPP-4 may yield truncation products that act upon GCGR expressed in α-cells. These findings are however unlikely to be pertinent for the glucagonostatic actions of most GLP-1RA, which are mostly completely DPP-4-resistant. While GLP-1 increases somatostatin secretion, δ-cells express GLP-1R at low abundance and indirect effects cannot be excluded. Finally, GLP-1R is present in the exocrine pancreas, localized to the acinar cells, where GLP-1 acts to increase digestive enzyme release.

The Brain GLP-1 system

Where does brain GLP-1 originate from?

Proglucagon (*GCG*) mRNA transcripts are detected predominantly in the brainstem (74, 75), whereas the glucagon-like peptides (including GLP-1), and the GLP-1R are more widely distributed throughout the central nervous system (CNS) (10, 75-80). Further characterization of the GLP-1-immunoreactive cells in the lower brainstem solidified the view that these cells are a specific population of neurons, rather than glial cells or endothelial cells (ECs), that are not catecholaminergic but primarily glutamatergic (79, 81). Additionally, the observation that GLP-1 immunoreactivity was localised to axon terminals in the hypothalamus further solidified the view that GLP-1 -cells in the brainstem are neurons and utilise GLP-1 as a neurotransmitter that is stored until release in the axon terminals. A first proof that this assumption is correct was provided by the observation that injection of a virus encoding shRNA against PPG into the NTS causes a significant decrease in GLP-1 immunoreactivity in the paraventricular nucleus of the hypothalamus (82). Functional studies in the rat combining immunohistochemical identification of these neurons with c-Fos immunodetection revealed their activation by malaise, stress and gastric distension and linked them to the suppression of food intake (83-86).

481 The GLP-1-producing neurons are also called preproglucagon (PPG) neurons or GCG neurons.
482 For the purpose of this review, the term PPG neurons will be used, independent of species, to
483 describe the neurons that produce GLP-1 within the CNS. These are largely equivalent cell
484 populations in higher mammals (87, 88). CNS GLP-1 activity has also been described in other
485 vertebrates, such as fish and bird, but specific accounts of PPG neurons in their brainstem are
486 lacking (89, 90).

487 The ability to interrogate the function of the CNS GLP-1 system was enabled by development
488 of transgenic mice targeting the GLP-1-producing neurons using endogenous *Gcg* promoter
489 sequences (91). The first mouse strain allowed fluorescent visualization of these cells in vitro
490 and thus electrophysiological studies of these neurons in brain slices (92). Additionally, the
491 strong expression of yellow fluorescent protein (YFP) in the cytoplasm of PPG neurons allowed
492 visualization not only of the cell body and terminals of cells enabling *Gcg* transcriptional activity,
493 but of the entire dendritic and axonal structure of these cells. These studies provided further
494 characterization of the main population of PPG neurons in the nucleus tractus solitarius (NTS)
495 and in the intermediate reticular nucleus (IRT), and the population in the olfactory bulb that was
496 previously detected by in situ hybridization (ISH) (93-95) (**Figure 2**). Additional PPG neurons
497 were located in the piriform cortex and in the lumbar-sacral spinal cord (93, 96). Widespread
498 projections throughout the brain were confirmed in unprecedented detail by revealing not only
499 terminal fields, that can be visualised with GLP-1 immunoreactivity, but also axons in passage.
500 Importantly, these transgenic mice established that hippocampus and most areas of the cortex
501 do not receive GLP-1 projections, findings with implications for understanding the mechanisms
502 of neuroprotection that have been postulated for physiological GLP-1 action (97). Subsequent
503 studies utilized mice with Cre-recombinase under the control of the *Gcg* promoter thereby
504 facilitating functional manipulation of these neurons in vivo (98-102). Importantly, these mice

allowed the selective ablation of NTS-PPG neurons and thereby the demonstration that loss of these neurons substantially decreased GLP-1 content in both hypothalamus and spinal cord (100) and thus corroborated the findings from rat, that interference with GLP-1 translation in the brainstem affects GLP-1 content in the forebrain (82). The 'second generation' *Gcg*-Cre transgenic mice additionally provided insights where Cre-dependent viral expression of Channelrhodopsin2-Green Fluorescent Protein (ChR2-GFP) was used to confirm the assumption that NTS-PPG neurons project to most (if not all) forebrain targets (103). These findings confirmed results of prior studies utilizing injection of retrograde tracers into various forebrain areas and co-localizing the tracer with GLP-1 immunoreactivity in the NTS and IRT (79, 104).

Manipulation of PPG neurons to interrogate their function in vivo

Studies in mouse and rat established that GLP-1 is produced by PPG neurons and is found in their cell bodies and axon terminals. Functionally, experiments using shRNA to knockdown GLP-1 expression produced a mild hyperphagic effect, mainly limited to rats on a high fat diet (82). Subsequent studies making use of transgenic mice with virally mediated Designer Receptors Exclusively Activated by Designer Drugs (DREADD) expression in the NTS PPG neurons, demonstrated that selective activation of these neurons produces a strong hypophagic effect (99-101).

This was followed by a study employing both inhibitory DREADD receptor expression as well as selective ablation of NTS PPG neurons by the expression of diphtheria toxin A subunit (DTA) to examine effects on food intake and brain GLP-1 content (100). Both hypothalamic and spinal cord GLP-1 content was severely reduced upon ablation of these neurons, thus demonstrating that brainstem GLP-1 neurons are the principal source of GLP-1 within the CNS and that GLP-1 is stored in the axon terminals of the PPG neurons, all of which have their cell bodies in the

lower brainstem. Interestingly, as observed before with shRNA knockdown in rat, there was little effect on basal food intake, but reduction of brainstem *Gcg* expression or ablation of PPG neurons impaired control of post fasting food intake and stress induced feeding reduction (82, 100).

These findings, together with the phenotype of normal food intake and body weight observed in $Gcg^{Gut-/-}$ mice with intestine-specific deletion of the proglucagon-derived peptides (PGDPs) (14), clarify the modest physiological importance of either GLP-1 derived from PPG neurons or the gut for control of food intake. Moreover, neither chemogenetic activation, nor ablation of CNS PPG neurons or knockdown of brainstem *Gcg* mRNA affected glucose tolerance in regular chow fed mice or rats (82, 100, 102). Interestingly, shRNA knockdown of GLP-1 expression from PPG neurons in HFD-fed rats resulted in moderately impaired glucose tolerance, whereas chemogenetic activation of PPG neurons in HFD-fed mice did not impact glucose tolerance. Intriguingly, correlation of NTS levels of *Gcg* mRNA with fat mass was observed in rats, implying signals linking the extent of adiposity to central PPG activity.

ICV injection of the GLP-1R antagonist Ex9 in chow-fed rats and mice impaired glucose tolerance (82, 105), but ablation of PPG neurons in chow-fed mice did not impair glucose tolerance (100). The simplest explanation for this discrepancy would be that PPG neurons are not involved in the regulation of glucose tolerance, but that gut-derived GLP-1 is able to reach (parts of) the brain to affect glucose tolerance, or that ICV Ex9 actually accesses the general circulation and exerts direct effects, e.g. in the pancreas. Alternatively, basal constitutive GLP-1R signaling may exert tonic control of these processes, which may be disrupted by pharmacological administration of GLP-1R antagonists independent of the source of the GLP-1 ligand (106-108). Finally, chemogenetic activation of PPG neurons increases heart rate, but their inhibition or ablation has no effect on resting or active heart rate (HR) or blood pressure

(BP) (109). All these results point at the central GLP-1 system as being capable of exerting significant effects on food intake and on autonomic nerve activity, but not producing these effects tonically in daily life.

What is the physiological role of brain-derived GLP-1?

These findings raise multiple questions, namely how important is brain-produced GLP-1 for physiology, how is it regulated and how and where does it act in the CNS? Is its regulation linked to circulating GLP-1? Interpretation of these findings requires careful scrutiny of the specific diets, age and sex of the animal species, housing conditions, and experimental manipulations utilized to infer conclusions about CNS GLP-1 action. For example, the actions of Ex9 to increase food intake were observed in satiated but not in hungry rats (110). Similarly, multiple studies have employed scheduled meals to observe CNS effects of GLP-1 on food intake (107, 111, 112). GLP-1 and GLP-1Rs within the brain may not be critical for feeding under ad libitum access to food, but only under conditions where a large meal is consumed, such as after a prolonged fast, or in the case of sporadic availability of highly palatable food to these rodents. A likely contributing signal here is the extent of gastric distension. Notably, c-Fos activation in NTS PPG neurons requires a higher degree of gastric distension, then for instance c-Fos activation in neighbouring catecholaminergic neurons (83, 85).

PPG neurons may also have developed as a risk assessment system to balance predatory risk (manifested as stress leading to meal termination) with the need for food/energy intake. Progressive gastric distension may shift activity away from intake towards the avoidance of predation as the stomach becomes full and a higher percentage of PPG neurons becomes activated (84). Simultaneously, PPG neurons are also activated in situations of stress, be this psychological or restraint stress or consumption of a toxic substance, as mimicked experimentally by injection of lithium chloride LiCl or lipopolysaccharide (LPS) (104, 113).

Additionally, PPG neurons are activated by local (NTS) availability of leptin or cholecystokinin (CCK), as well as electrical stimulation of vagal terminals in the NTS (92, 114). Thus, PPG neurons are activated by signals linked to short- and long-term energy balance and can reduce food intake, but do not seem to play a major role in determining food intake and body weight in ad libitum fed rodents. Rather, their physiological role seems to be tied to the response to various forms to stress, but might lend themselves to be exploited clinically to achieve hypophagia and weight loss. A role for PPG neurons beyond the monitoring of short- and long-term energy balance was also suggested by a comprehensive study of their mono- and polysynaptic inputs within the central and peripheral nervous system (115). The localization of GLP-1Rs that serve to mediate the actions of GLP-1 released from PPG neurons is discussed below.

GLP-1 receptors within the nervous system

Various techniques, employing radiolabelled or fluorescent GLP-1R agonists or antagonists, or specific GLP-1R antisera, together with ISH, have localized GLP-1R expression within the nervous system (49, 116-120).

Complementary studies have used mice with germline or conditional tissue-specific deletion of the GLP-1R to examine the importance of different GLP-1R populations in the CNS and periphery (121-126). Notably, the hypophagia caused by ICV injection of GLP-1 was fully abrogated in *Glp1r*$^{-/-}$ mice, consistent with the loss of binding sites for Ex4 in the *Glp1r*$^{-/-}$ mouse brain (121). These findings align with the demonstration that PPG neuron activation produces hypophagia, but that the loss or attenuation of PPG neuronal activity or genetic deletion of gut GLP-1 does not cause overeating or obesity (14, 100-102, 127). Thus, although GLP-1 has an

600 essential role in the control of blood glucose, it is dispensable for the suppression of food intake
601 at least under standard animal house holding conditions.

602 GLP-1Rs are expressed throughout the CNS, from the olfactory bulb down to the spinal cord
603 (35, 75, 128, 129) (**Figure 3**). The use of genetic targeting approaches has revealed specific
604 cell types producing various GLP-1 responses. Adams et al. deleted the GLP-1R from either
605 vGLUT2 or vGAT-expressing cells, thus removing the receptor from glutamatergic and
606 GABAergic neurons, respectively. Only those mice lacking GLP-1R in glutamatergic cells lost
607 the hypophagic and long term body weight-reducing effect of liraglutide, suggesting that
608 liraglutide affects food intake and body weight via GLP-1Rs located on glutamatergic, but not
609 GABAergic cells (125). In contrast, Fortin et al. used Glutamic Acid Decarboxylase (GAD)-Cre
610 rats to target GABAergic neurons (130). Their strategy was to virally express an inhibitory
611 DREADD receptor in GAD-expressing cells in the NTS and to activate this receptor in the
612 presence of liraglutide. Inhibition of NTS GABAergic neurons prevented the anorectic effect of
613 liraglutide. They concluded that GABAergic neurons in the NTS mediate the hypophagic effects
614 of liraglutide. Reconciling these studies in mice and rats, Adams et al. demonstrated key roles
615 for GLP-1Rs on glutamatergic but not GABAergic neurons yet they did not show that GABAergic
616 neurons per se are not required for the feeding response. Similarly, Fortin et al. demonstrated
617 that the activity of GABAergic neurons in the NTS is required for the hypophagic response to
618 liraglutide, but they did not prove that it is actually the GLP-1R expressed on these neurons
619 that is required for the response. Thus, one might hypothesize that liraglutide binds to GLP-1Rs
620 on glutamatergic neurons, and these in turn signal to GABAergic neurons and their release of
621 inhibitory transmitter is required for the suppression of food intake. Hence, caution is needed
622 when using chemogenetic approaches to extrapolate the precise physiological pathways
623 employed by GLP-1 within the CNS.

Neuronal GLP-1Rs outside the blood-brain barrier

GLP-1Rs on vagal afferent neurons

GLP-1Rs are expressed by sets of vagal afferent neurons (35, 131, 132), which are mechanoreceptors (133). It has been tempting to assume that these GLP-1R expressing neurons are those stretch-activated vagal afferents that activate PPG neurons (85). However, a study examining this hypothesis found that GLP-1R expressing vagal afferent neurons represent only a very small fraction of the vagal afferent inputs to PPG neurons (135). GLP-1R-expressing vagal afferent neurons have their sensory inputs within the gastrointestinal tract, their cell bodies in the nodose ganglion, and their axon terminals in the NTS; where exactly their GLP-1Rs are located, remains unclear. Thus, the source of GLP-1 activating these neurons might be the gut-derived GLP-1 for the peripheral nerve endings, or the PPG neuron-derived GLP-1 if their central terminals express GLP-1R. The physiological role of GLP-1Rs expressed in vagal afferent neurons was examined by viral delivery of shRNA targeting the nodose ganglion (NG) GLP-1R in male Sprague Dawley rats (134). GLP-1R knockdown in vagal afferent neurons did not affect long term energy balance but did accelerate gastric emptying, findings consistent with the phenotype of mice with genetic reduction of *Glp1r* expression using *Phox2b*-Cre to target autonomic neurons including the NG (126). Reduction of NG *Glp1r* expression also blocked the effects of intraperitoneal, but not intraventricular, Ex4 on food intake and gastric emptying in rats (134), whereas the sustained actions of large GLP-1RA such as dulaglutide to generate weight loss were also modestly attenuated in mice with genetic reduction of NG *Glp1r* expression (126). Collectively, these observations imply that the GLP-1Rs expressed by vagal afferent neurons are located in the periphery, outside the blood-brain barrier, rather than on the nerve terminals inside the NTS. Thus, GLP-1Rs expressed by

vagal neurons seem to be a physiological target for gut-derived or pharmacologically administered GLP-1.

GLP-1Rs in circumventricular organs

Similar uncertainty arises in the context of interrogating the importance of GLP-1Rs expressed by neurons in the circumventricular organs that lack a functional blood brain barrier. These regions, such as the area postrema (AP), median eminence (ME) and part of the neighbouring arcuate nucleus (ARC), Subfornical organ (SFO) and organum vasculosum of the lamina terminalis (OVLT), are accessible from the circulation, and thus are potential targets for post-prandially released gut-derived GLP-1 as well as confirmed targets for systemically-administered GLP-1RAs (**Figure 3**). However, some of these regions are also innervated by PPG neurons (93). The two circumventricular regions that have been investigated in some detail are the ME/ARC and the AP. Whilst systemic administration of GLP-1RA leads to activation visualised by c-Fos immunoreactivity, in many areas of the brain, it is particularly these areas where clear binding of fluorescent GLP-1RA is observed (116-118).

Most likely, engagement of GLP-1Rs in these areas, as indirectly visualised by c-Fos staining in GLP-1R+ cells (125), leads to additional secondary c-Fos activation along downstream pathways in the CNS (116-118). Experiments employing physical lesions to destroy the AP, the ARC or the PVN, as well as subdiaphragmatic vagal afferent deafferentation (SDA) indicated that the majority of the hypophagic effect of the GLP-1RA liraglutide resides within the ARC (118). Chronic infusion of Ex9 into the PVN, but not the ARC, increased body weight (118), however, Ex9 reduced the body weight-lowering effect of liraglutide when administered into the ARC, but not the PVN. These results indicate that whilst the ARC may be a key target for the anorectic actions of liraglutide there is no tonic activation of the ARC GLP-1Rs by endogenous GLP-1 released either by the gut or the PPG neurons.

The available evidence distinguishes between the weight lowering effects of systemically administered GLP-1RA from those of PPG neuronal activation. This notion is supported by studies dissociating the hypophagic effects of PPG neuron activation from the food intake suppressing actions of subcutaneous semaglutide (135). It seems possible that GLP-1Rs expressed in the ARC may be activated by high circulating levels of GLP-1 under conditions of severe intestinal malaise or following bariatric surgery. This distinction may explain why GLP-1RA therapy is associated with an increased sensation of nausea (136), whilst chemogenetic activation of brainstem PPG neurons does not elicit adverse responses in mice (102, 135).

GLP-1Rs inside the blood-brain barrier

Most other GLP-1R-expressing brain regions receive axonal inputs from PPG neurons, and are likely activated by GLP-1 originating from those cells. However, brainstem PPG neurons do not project to the hippocampus or to the cerebral cortex (93). Thus, it is currently unclear how GLP-1Rs located in these areas are activated. A small population of PPG neurons has been reported in the piriform cortex (93), but these do not give rise to axons in other parts of the cortex or the hippocampus. Activation of GLP-1Rs in the ventral hippocampus also results in hypophagia, and it has been suggested that volume transmission from the lateral ventricles, which had detectable levels of GLP-1, might account for their physiological activation (137). These observations are consistent with the possibility that PPG neurons might be the source of GLP-1 within the ventricle, as PPG axons are found along the ependymal cell layer covering the surface of the ventricles and intraventricular injection of a retrograde tracer labelled a fraction of NTS PPG neurons (137). Whilst this is plausible, it should be noted, that both GLP-1RA and GLP-1R antagonists given subcutaneously access the ventricular space (49, 116, 118). Hence, there is no discernable barrier for pharmacological or gut-derived GLP-1 to reach the brain ventricles, but it does not provide evidence that post-prandially released GLP-1 would

meaningfully access the ventricles. Additionally, the observation that there is very little entry of systemically administered GLP-1R agonists into the brain parenchyma beyond the circumventricular organs, and particularly into the ventral hippocampus, suggests that there is no free diffusion between the ventricular space and the brain parenchyma (116, 118). This would then argue against PPG neuron-derived GLP-1 entering the ventricles and exiting at the level of the ventral hippocampus. A final area that is not innervated by brainstem PPG neurons is the olfactory bulb. However, the olfactory bulb contains both GLP-1-and GLP-1R-expressing cells (75, 95, 138), and could thus constitute an independent local GLP-1 system. Whether or not the olfactory bulb is accessible to GLP-1 from the general circulation is not clear; no studies utilizing GLP-1R agonists or antagonists to date have visualized binding in the region of the olfactory bulb.

When interrogating the function of GLP-1R populations within the brain, modulation of food intake is often the primary measured output, but the physiological mechanisms underlying modulation of this output varied substantially between studies and brain regions. For example, food intake might also be reduced due to a reduction in the reward value of the specific substance tested, findings potentially relevant for the actions of substance abuse, the sensation of nausea or other malaise, or the presence of acute or chronic stress (139-142). Additionally, neural GLP-1R activation has been linked to an increased cardiovascular output, or to changes in body temperature, or increased peripheral brown fat activity, all likely consequences of activation of the sympathetic nervous system (143-146) and therefore in line with the proposed functional role of PPG neurons in stress responses as discussed above.

Concluding remarks about GLP-1R expression and GLP-1 action in the nervous system

The emergence of fluorescently labelled GLP-1RA and transgenic mouse models combined with the use of viral vectors to dissect the brain GLP-1 system over the past decade has

highlighted our lack of deep understanding of GLP-1 action in the brain. It is now emerging that there is no direct link between the gut and brain GLP-1 system, and that GLP-1RAs exert their appetite suppressing effects primarily, if not exclusively by interacting with GLP-1Rs on cells in the circumventricular organs outside the blood-brain-barrier, that then signal deeper into the brain but do not involve activation of PPG neurons, the brain's own source of GLP-1. Understanding the physiology of brain-derived GLP-1 is even more nascent and unravelling the details of its regulation and various actions including, but not limited to, the reduction in food intake, promises to be an exciting journey of discovery.

GLP-1R expression in the cardiovascular system

Cardiovascular safety trials with GLP-1RA have demonstrated reduction in all cause- and cardiovascular mortality, and lower rates of major cardiovascular events including stroke and myocardial infarction (15, 16). GLP-1RA also increase HR in humans and animals, findings attributed in part to activation of the sympathetic nervous system, inhibition of the parasympathetic nervous system, and activation of the sinoatrial GLP-1R (143, 144, 147, 148). GLP-1R signaling also attenuates the extent of experimental stroke in preclinical studies (97), and GLP-1RA reduce the rates of stroke in humans with risk factors for or established cardiovascular disease (149). Furthermore, GLP-1RA also reduce albumin excretion, and trials are underway to determine whether these agents may reduce rates of dialysis, decline of eGFR and renal replacement, in people with diabetic kidney disease. Collectively, these observations have created great interest in understanding how GLP-1RA exert their mechanisms of action in the cardiorenal system. Nevertheless, there is still relative uncertainty and some controversy about the precise cell types and tissues that express the GLP-1R in the heart, peripheral vasculature, and kidney (**Figure 4**).

743 ***Localization of GLP-1R in the heart***

744 Despite considerable effort, the precise identity of GLP-1R+ cells in the heart remains elusive.
745 (44). Analysis of hearts from 3 cynomolgus monkeys, including sections from males and
746 females, revealed GLP-1R-immunopositive cells using mAb 3F52 only in sinoatrial myocytes,
747 which were identified by co-staining sections for hyperpolarization activated cyclic nucleotide
748 gated potassium channel 4 (HCN4). In a single frozen sample taken adjacent to the SA node
749 confirmed to be GLP-1R immunopositive by IHC, ^{125}I-GLP-1 binding was detected and
750 diminished following addition of excess unlabeled ligand. GLP-1R-immunopositive cells were
751 also visualized in sections from the sinoatrial node from a single normal human heart (44).

752 Wallner *et al.* reported *GLP1R* expression in right atria (RA) and right ventricle (RV) tissue from
753 7 human heart samples that were not suitable for transplantation. Relative *GLP1R* expression
754 by qPCR was 3.4-fold higher in the RA compared to the RV (150). *GLP1R* mRNA transcripts
755 were also amplified by conventional PCR generating a 103 base pair (bp) cDNA product from
756 RNA isolated from the RA, RV and from 3 samples of isolated cardiomyocytes from the left
757 ventricle (LV). Clarke and colleagues analyzed GLP-1R protein expression in the human heart
758 by IHC using Mab 3F52 to analyze cardiac tissue obtained from anonymous donors from two
759 tissue biobanks (151). Extensive GLP-1R-immunopositivity was reported in atrial and
760 ventricular cardiomyocytes, but not within ECs or vascular smooth muscle cells (VSMs). The
761 proportion of GLP-1R+ cells, and the number of hearts or sections examined was not described
762 (151). Giblett and colleagues from the same research group also analyzed GLP-1R protein
763 expression in human heart tissue obtained from non-diabetic human subjects with ischemic
764 heart disease using mAb 3F52 (152). Patchy GLP-1R immunoreactivity was observed in
765 cardiac myocytes from the right and left ventricle (RV and LV, respectively), however the

precise location of the GLP-1R-immunopositive cardiomyocytes was not described, and the proportion of GLP-1R-immunopositive cells detected within each analyzed heart was not reported (152).

GLP1R expression was examined in RNA samples from all 4 chambers of 15 healthy and diseased human hearts by qPCR, and RT-PCR for the full length mRNA transcript (18). *GLP1R* mRNA transcripts were detected in all of the hearts examined, with similar levels in RA vs. RV but lower levels in the LV vs the LA. The relative abundance of the *GLP1R* in heart tissue was similar to that detected in human islet RNA samples. Nevertheless, the cellular localization of GLP-1R expression in the heart, assessed using both ISH and IHC with Mab 3F52, was not identified in the majority of sections analyzed, except in the RA, where *GLP1R* RNA was detected by ISH (18). Moreover, GLP-1R protein was not detectable in human cardiac tissue by Western blot analysis using Mab 3F52, although this antibody did recognize the GLP-1R protein in extracts subjected to immunoprecipitation and immunoblotting from cells transfected with a cDNA encoding the *GLP1R*. The discrepant results for GLP-1R localization in the human heart using the same antibody in various reports likely reflect different technical conditions and controls employed for IHC in various laboratories.

Analysis of *Glp1r* expression in the mouse heart reveals levels of *Glp1r* mRNA transcripts are higher in the atria than ventricles. PCR amplification of *Glp1r* transcript in 3 samples from normotensive or hypertensive angiotensin II-infused mice detected *Glp1r* mRNA transcripts in atria however much lower levels were detected in ventricular tissue (153). Whether this discrepancy reflects true-species differences or region-specific differences in RNA sampling of human ventricular biopsies is unclear as comparable levels of *GLP1R* mRNA transcripts were

detected in RNA extracted from biopsies obtained from all 4 chambers of the human heart (18). Consistent with *Glp1r* expression in an atrial myocyte-like cell type, *Glp1r* expression was reduced in mouse atria using the cardiomyocyte specific αMyosin Heavy Chain (MHC) promoter to drive tamoxifen inducible Cre-ER recombinase in cardiomyocytes (147). To further determine the identity of mouse atrial GLP-1R+ cells, Baggio *et al.* used the *Hcn4* promoter, classically expressed in sinoatrial (SA) node cells, to drive Cre recombinase expression and specifically target SA gene expression (148). Although *Hcn4*-Cre reduced levels of *Gcgr* mRNA transcripts in control experiments using floxed *Gcgr* mice, no reduction of atrial *Glp1r* levels was achieved using *Hcn4*-Cre and floxed *Glp1r* mice. Hence, the precise atrial cell type(s) that express the murine cardiac GLP-1R remain uncertain.

Assessment of GLP-1R expression in blood vessels

Despite the multiplicity of studies describing actions of GLP-1RA on blood vessels, there is surprisingly little rigorous information that describes GLP-1R expression in normal or diseased VSMs, ECs or other vascular cell types. Much of the data in this area relies on ECs and VSMs analyzed after cell culture ex vivo, which may not be representative of GLP-1R expression in the same cell types in vivo. Indeed *GLP1R* mRNA transcripts were not detected by qPCR in RNA isolated from human coronary artery VSMs or ECs (18). However, Richards reported *Glp1r* promoter-directed fluorescent cells in the aorta, and arteries and arterioles of multiple tissues, and *Glp1r* mRNA transcripts were detected by qPCR in mouse aorta, albeit at levels 90% lower than corresponding expression of *Glp1r* in RNA isolated from mouse atrial tissue (35). Fluorescent cells within the intestinal vasculature co-stained with α-smooth muscle actin (αSMA) and Neural/glial antigen 2(NG2), a marker for pericytes, and were thought to represent

arterial, rather than venous blood vessels. Nevertheless, *Glp1r* expression was not examined in the majority of cells+ for the fluorescent reporter.

GLP1R mRNA transcripts were detected by qPCR in the aortic intima and media obtained from subjects, with and without obesity, scheduled for aortic surgery (154). Extensive diffuse GLP-1R-immunopositivity was detected in the aorta using Mab 3F52. Notably, *GLP1R*/GLP-1R expression was not quantified relative to other *GLP1R*+ tissues, nor assessed using the same reagents and conditions for comparative purposes of specificity in organs known not to express the GLP-1R.

Single cell analysis of murine endothelial cells from 20 organs showed *Glp1r*+ cells most prominently in the lung (**Figure 5**), with some positive ECs also detected in cells from the liver and heart (155). Given the limitations of read depth needed to identify low abundance transcripts with scRNA-seq, this is likely a conservative snapshot of EC *Glp1r* expression. Consistent with *Glp1r* expression in ECs, mice expressing EC-specific *Cdh5*-dependent Cre recombinase exhibited reduced *Glp1r* expression in a lung EC preparation isolated using CD31 and CD102 coated beads (156). However, no markers were used to assess the extent of EC enrichment, and knockdown of *Glp1r* was not examined in resistance arteries, kidneys, heart, aorta or other vascular sites that might contain EC GLP-1R expression. Using *Glp1r*-Cre mice crossed with a ROSA26-tdRFP reporter, Richards and colleagues also detected *Glp1r*-directed reporter gene expression in a few blood vessels within the cardiac ventricles, with a morphology consistent with VSMs (35). Overall, the data suggests that *Glp1r*/*GLP1R* expression in the vascular system is highly heterogeneous, with high expression in mouse lung ECs and restricted expression in subsets of VSMs in the kidney (discussed next), rather than broadly

838 expressed throughout the vasculature.

839

840 ***GLP-1R expression in renal blood vessels***

841

842 The location of GLP-1R expression within the kidneys has been controversial, at times localized
843 to glomerular or tubular epithelium in studies using GLP-1R antisera that do not detect the GLP-
844 1R or lack specificity (11, 20, 43). *In vivo* autoradiography using ^{125}I-labeled GLP-1 (7-36)
845 amide, IHC using well characterized GLP-1R antibodies, and ISH have detected GLP-1R in the
846 rat, mouse and monkey renal vasculature, particularly in arterial VSMs (44, 157-159). Pyke *et*
847 *al* (44) analyzed kidney from 7 normal rhesus monkeys using Mab 3F52. GLP-1R-
848 immunopositivity was limited to a small number of VSMs within the preglomerular vascular
849 compartment. These findings were corroborated by analysis of matched sections using *in situ*
850 radiolabeled ligand binding, which identified binding in a vessel that also stained positive with
851 Mab 3F52 (44).

852

853 RT-PCR of RNA from isolated mouse kidney fractions, and ISH analysis of mouse kidney
854 detected *Glp1r* mRNA transcripts in blood vessels and not in the tubular fraction (157). A few
855 GLP-1R+ renin+ juxtaglomerular cells were reported in the afferent arterioles of the monkey
856 and mouse kidney (44, 158). Detection of GLP-1R immunopositivity in several VSMs within the
857 preglomerular vascular compartment was corroborated by analysis of matched sections using
858 *in situ* radiolabeled ligand binding, which identified binding in a blood vessel that also stained
859 positive with Mab 3F52 (44). Moreover, the same antisera failed to identify GLP-1R-
860 immunopositive cells in the kidney from *Glp1r*$^{-/-}$ mice. Despite detection of GLP-1R+ cells within
861 a subset of the renal vasculature, the majority of renal blood vessels and VSMs examined by

ICC or ISH do not express the GLP-1R, and studies using validated reagents have not detected the GLP-1R in glomerular epithelial or tubular cells (44, 157, 158). Hence, the mechanisms by which GLP-1 modulates renal function, including control of albumin, water and salt excretion, either directly through control of the renal vasculature, or via indirect mechanisms, remain uncertain.

GLP-1 production in enteroendocrine L cells along the gut

Enteroendocrine L cells have classically been defined as the cell type giving rise to GLP-1 in the gut (1). In humans and mice, the majority of L cells reside in the ileum and colon, with fewer cells located in the duodenum and jejunum. L cells sense various nutrients from the luminal surface and in turn secrete GLP-1 into the local circulation (160). Numerous cognate receptors for non-nutrient GLP-1 secretagogues have also been identified on the basolateral side of L cells, thus enabling L cells to simultaneously sense molecules in the circulation (161-163). L cells in the proximal gut are capable of responding briskly to nutritive stimuli (14, 164); the available data suggests that distal gut L cells exhibit secretory responses biased towards non-nutrient secretagogues (164). Analysis of mice with marked reduction of *Gcg* expression within the entire, or distal gut, reveals essential contributions of intestinal *Gcg* expression to the pool of circulating GLP-1, and in the control of gastric emptying and glucose homeostasis (14).

scRNA-seq analyses have illuminated the transcriptomic heterogeneity evident across subsets of L cells (**Figure 6**). One of the first scRNA-seq studies on the mouse small intestine recovered 310 out of 7216 (4.3%; which is higher than often achieved) quality control-passed EECs (165), and 10% of these were identified as *Gcg*+. Subsequently, several reports characterized

886 individual L cells isolated from mouse reporter lines. Sequencing of 259 individual YFP$^+$ cells
887 isolated from the small intestine of GLU-Venus mice identified three main subgroups of L cells.
888 These corresponded to 1) *Gcg*/*Pyy* double-positive cells, which account for 50% of L cells; 2)
889 *Gcg/Tph1/Pzp* triple-positive cells, which account for 35% L cells; and 3) *Gcg*/*Gip* double-
890 positive cells, which account for 15% L cells (166). Similarly, a comprehensive survey of 6,906
891 small intestine EECs from *Neurog3Chrono* reporter mice identified more than 90% of L cells as
892 multi-hormonal (167). The detection of *Gcg* in EECs correlated strongly with *Cck*, followed by
893 *Pyy*, *Ghrl*, and *Sct* co-expression (**Figure 6**). A scRNA-seq study on 1,560 intestinal YFP$^+$ cells
894 isolated from *Neurod1*-Cre; *Rosa26*-YFP mice confirmed that colonic L cells are also mostly
895 multi-hormonal (168). Although both small and large bowl L cells can produce neurotensin, L
896 cells in the distal colon preferentially expressed *Pyy* and *Insl5*. Studies of human EECs have
897 relied primarily on bulk RNA-seq for transcriptome profiling (169-171). To circumvent the
898 difficulty of isolating L cells from human intestine with high purity, a recent study sequenced
899 2,255 cells single cells from human small intestinal organoids transduced with doxycycline-
900 inducible *NEUROG3* transgenes to drive differentiation towards endocrine lineages (172).
901 Three L cell subtypes among the 2,255 EECs sequenced were *PYY*^{High}, *PYY*^{Low}, and *NTS*^{High}
902 cells, all of which were also detected in the mouse L cell scRNA-seq datasets. A discrepancy
903 between the mouse and human data was the absence of *TPH1*$^+$ and *GIP*$^+$ L cells, although the
904 number of organoid-derived L cells sequenced in the study was not high (< 100), and the L cell
905 subtypes in the organoid might not faithfully recapitulate the ones in the human intestine *in situ*.
906 Nevertheless, the analyses and corroboration of mouse and human L cell scRNA-seq datasets
907 support a conserved transcriptional program in L cells across the species. Analysis of human
908 jejunal L cell transcriptomics, and L cell density identified dysregulation of enteroendocrine
909 differentiation signatures associated with reduced L cell density in obese people with T2D (173),

providing a possible explanation for reduced meal-stimulated levels of GLP-1 in some of these individuals. Collectively, these findings raise several important questions that warrant further interrogation; do different L cell subtypes produce and secrete GLP-1 in a secretagogue-selective manner with different secretory capacity? Can we attribute the functional heterogeneity of L cells predominantly to their transcriptional signatures or does their location within the gut, or along the crypt-villus axis, cellular neighborhood and co-existing disease states modify their phenotype? Are there new molecular targets that could be identified from these datasets for augmenting endogenous GLP-1 secretion?

GLP-1R expression in the gastrointestinal tract

GLP-1R expression is detected across the proximal and distal regions of gastrointestinal (GI) tract. The close proximity of intestinal GLP-1R-expressing immune cells and enteric neurons to L cells within the intestine may facilitate immediate paracrine actions of GLP-1. Remarkably, despite robust actions of GLP-1RA to reduce enterocyte chylomicron synthesis and secretion, the canonical GLP-1R is not detectable within the small or large bowel enterocytes. Below we highlight regional considerations and GLP-1R+ cell types within the gut.

GLP-1R expression and biology in gut intraepithelial lymphocytes

Intestinal intraepithelial lymphocytes (IELs) are a unique population of T cells with innate-like immune function. Percoll-purified IELs express high levels of *Glp1r* relative to the thymus, spleen, and lymph nodes as shown by qPCR (174). Furthermore, IELs from the small bowel express higher levels of *Glp1r* than colonic IELs. Whole body loss of the *Glp1r* does not perturb the number and composition of IELs (174). The IEL GLP-1R is functional, as Ex-4 directly

augments cyclic adenosine monophosphate (cAMP) levels in murine gut IELs *ex vivo* (174). Within the intestinal epithelium, both TCRγδ^{+} and TCRαβ^{+} IELs express comparable levels of *Glp1r* as shown by qPCR on sorted IELs (175). The importance of gut IEL GLP-1Rs for regulating incretin biology and metabolism has been examined in the context of β7$^{-/-}$/ mice, which lack gut IELs due to defects in lymphocyte gut homing (175). Reconstitution of irradiated *Ldlr*$^{-/-}$ mice with a chimera of β7$^{-/-}$/*Glp1r*$^{-/-}$ bone marrow raised plasma GLP-1, improved intraperitoneal glucose tolerance, lowered plasma cholesterol, and reduced aortic plaque size after 14 weeks of high-fat high-cholesterol diet feeding, relative to irradiated WT mice with a chimera of β7$^{-/-}$/WT bone marrow. How gut GLP-1R-expressing IELs control the bioavailability of local and systemic GLP-1 remains uncertain (175). The physiological importance of the enteroendocrine-IEL axis for local and systemic immunity remains incompletely defined.

Stomach and Brunner's glands express the GLP1R

Human gastric mucosa and parietal cells express *GLP1R* mRNA transcripts as detected by RT-PCR (176) and multiple GLP-1R-immunopositive cells were detected within the gastric epithelium using a partially characterized monoclonal antibody Mab 28141, findings consistent with the detection of *Glp1r* transcripts in rat parietal cells (177). GLP-1 may act on the parietal cell GLP-1R to block gastric acid secretion (178, 179), however few studies have explored the function of GLP-1R signaling within parietal cells *in vivo*.

Brunner's glands, found within the proximal duodenum, are a set of submucosal GLP-1R+ exocrine glands specialized in secreting mucus. The glands were highly GLP-1R-immuno-reactive using validated GLP-1R antibodies in both mice (using Mab 7F38) and in humans (using Mab 3F52) (44, 119). CD-1 mice injected with a single dose of Ex4 (1 mg/kg) showed

upregulation of *Il33* and *Ccl20* in RNA isolated from Brunner's glands (180), however the physiological importance of the Brunner's gland GLP-1R system remains poorly defined.

GLP-1R expression in the enteric nervous system

The enteric nervous system (ENS) is unique in its capability to transduce signals somewhat independently from the brain or the spinal cord (181). It comprises three main cell types: enteric neurons, enteric glial cells, and interstitial cells of Cajal. These cells cluster in the myenteric plexuses, found in the muscle layer of the gut, and in the submucosal plexuses, found in the submucosal layer of the gut, along the entire gastrointestinal tract (181).

Multiple lines of evidence demonstrate GLP-1R expression in subsets of enteric neurons. A study using mouse reporter lines expressing *Rosa26*-tdRFP or *Rosa26*-eYFP under the control of *Glp1r*-Cre found a small number of fluorescent-positive enteric neurons (35); treatment with 10 nmol/L GLP-1 increased their action potential firing frequency *ex vivo*. About 60% and 19% of these fluorescent-positive neurons from the small and large intestine, respectively, were positive for neuronal nitric oxide synthase, implying that these neurons are likely inhibitory motor neurons (35). Complementary analyses employed *Wnt1*-Cre to knock down *Glp1r* in the mouse central and enteric nervous system (126). *Glp1r* mRNA transcripts were more abundant in the muscle layer (including the myenteric plexus) than in the submucosal layer (including the submucosal plexus). *Wnt1*-Cre enabled knockdown of more than 95% of *Glp1r* mRNA in the ileal muscle layer, but only 80% in the jejunal and as low as 60% in the duodenal muscle layer (126). Human myenteric plexuses were reported to contain GLP-1R immunoreactivity however these experiments utilized an antibody, Ab39072 subsequently shown to lack sensitivity and

specificity (20, 182). A single cell atlas of the murine (and human) ENS affirmed the presence of *Glp1*r/*GLP1R* mRNA transcripts in various neuronal subsets within the ENS (**Figure 7**) (183). Beyond enteric neurons, it remains unclear whether enteric glial cells and interstitial cells of Cajal express the *Glp1r*.

The role(s) of GLP-1R signaling in enteric neurons have not been fully elucidated. Inference from the scRNA-seq data suggests that the GLP-1R-expressing enteric neurons are secretomotor/vasodilator neurons. Analysis of mice expressing a novel *Glp1r*-Cre; *lox-tdTomato* reporter demonstrated tdTomato-positive neurons lining the stomach and the intestine – these neurons were identified as mechanosensitive and were not involved in nutrient detection (133). Consistent with these findings, mice lacking *Glp1r* in their enteric neurons exhibited normal food intake, gastric emptying, body weight, and glucose control (126), suggesting that GLP-1R expression in enteric neurons is physiologically dispensable for the classical metabolic actions of GLP-1 and some aspects of nutrient sensing. Notably, adenosine triphosphate (ATP) co-released with GLP-1 from L cells, might also trigger vagal activation through $P2Y_{2/3}$, adding further complexity to L cell-ENS communication (184)

Potential GLP1R expression in intestinal smooth muscle

Whether GLP-1R agonism inhibits gut motility indirectly through neural mechanisms, or in part through direct interaction with a smooth muscle GLP-1R remains unclear (185-187). GLP-1 may also reduce smooth muscle cell inflammation in the context of lipopolysaccharide-induced *Tnf* and *Il1a* expression in colonic smooth muscle cells isolated from BALB/c mice (188). Notably, ascertainment of definitive non-vascular smooth muscle cell GLP-1R expression

remains challenging to date, as contamination from rare cell types within *ex vivo* preparations, including enteric neurons, cannot be excluded.

Thus, the local gut GLP-1 system is comprised of three main components: L cells as the main GLP-1 producer, and enteric neurons and IELs as the primary responders to GLP-1. Although GLP-1 secretion by L cells contributes to a majority of active GLP-1 levels in the circulation, it is worth mentioning that the local gut environment is frequently exposed to very high levels of GLP-1 (likely higher than the circulatory levels), especially when L cells are stimulated after meal ingestion or under various stress conditions. One can hypothesize that the presence of GLP-1R-expressing enteric neurons and IELs may present a feedback mechanism to modulate GLP-1 levels, as recently suggested (175). Moreover, local GLP-1 bioactivity is further modified by vascular and potentially enterocyte DPP-4 activity (189, 190). As enteric neurons and IEL subpopulations are difficult to isolate and study, the actions of GLP-1 on these cell types remains incompletely understood.

GLP-1 action in the immune system

GLP-1R agonists reduce systemic inflammation in preclinical and clinical studies, however the mechanisms linking GLP-1R signaling to modulation of immune cell activity remain uncertain (191). Low levels of *Glp1r* transcripts have been detected in isolated thymocytes, splenocytes, and lymph node cells from C57BL/6J mice by RT-PCR (192).

Glp1r expression was also detected by RT-PCR in murine T cells, both in the basal state and after stimulation with aCD3/CD28, as well as in TH1 and TH17 polarized cells by RT-PCR, however levels were not compared relative to *Glp1r* expression in other cell types or tissues

(193). Whole body *Glp1r*$^{-/-}$ mice display normal cellularity and immunophenotypes in the thymus, spleen, lymph nodes and bone marrow, suggesting that expression of *Glp1r* is dispensable for the normal development of these immune organs (192). Recent studies have begun to explore the biology of GLP-1R expression in tissue-resident immune cells.

Invariant natural killer T cells as targets of GLP-1 action

Invariant natural killer T (iNKT) cells exhibit properties of T cells and NKT cells; iNKT cells recognize the antigen-presenting CD1d that binds a variety of foreign lipids (194). A case report examined the putative direct effects of GLP-1 on human iNKT cells in the context of psoriasis. Two individuals with T2D and psoriasis treated with liraglutide exhibited reduced number of circulating iNKT cells after six weeks of treatment (195). Flow cytometric analyses revealed that 55% of CD3^{+} T cells and 96% of TCR-Vα24^{+} iNKT cells showed positive intracellular staining using an unspecified PE-GLP-1R antibody, accompanied by increased levels of cAMP levels in these cells following incubation with GLP-1. Exogenous liraglutide treatment of high fat diet (HFD)-fed mice for five days reduced the number of iNKT cells in the blood and adipose tissues (196). Moreover, HFD-fed *Cd1d*$^{-/-}$ mice, which lack iNKT cells in the body, lost less body weight when treated with liraglutide. Nevertheless, the expression of the GLP-1R in mouse iNKT cells was not definitively established and there is little rigorous evidence that B cells, NK, NKT, or innate lymphoid cells express the canonical GLP-1R.

GLP-1R expression in myeloid lineages

Immunoreactive GLP-1R protein has been detected in mouse peritoneal macrophages by Western blotting using incompletely characterized antisera (197, 198), and in macrophage-like cell lines. However, full-length *Glp1r* transcripts have not been consistently detected in macrophages from mouse tissues or peritoneum, in the basal state or after induction of inflammation (20). Flow cytometry was used to investigate GLP-1R expression in peripheral blood from individuals with allergic asthma (199). About 10% and 5% of blood neutrophils and eosinophils, respectively, from healthy control subjects stained positive with an Alexa Fluor 700-conjugated GLP-1R antibody. The proportion of GLP-1R-immuno-positive eosinophils was reduced to 2% in subjects with asthma. The specificity of the GLP-1R antibody used in this study, AF700-GLP-1R, remains uncertain. Overall, the expression of GLP-1R has not yet been robustly confirmed in myeloid cells.

GLP-1R transcripts in miscellaneous tissues

Adipose tissue

GLP1R transcripts are reported in adipocyte cell lines ex vivo and in human visceral and subcutaneous adipose tissues by Taqman-based qPCR (200). Nevertheless, the precise GLP-1R+ cell type(s) within rodent or human adipose tissue, ranging from vascular ECs or VSMs, immune cells, or adipocytes themselves, has not been defined with certainty.

Iacobellis *et al.* detected *GLP1R and GLP2R* RNA by RNA-seq and qPCR in 8 human epicardial adipose tissue (EAT) samples from individuals with coronary artery disease with and without T2D (201). *GLP2R* mRNA transcripts were ~5-fold more abundant than *GLP1R* mRNA, *GLP1R* expression was described as “relatively low” however precise quantification or comparison with

GLP1R expression in other tissues was not provided. GLP-1R-immunopositivity was detected in EAT and subcutaneous adipose tissue (SAT) from a diabetic and non-diabetic subject, analyzed by fluorescent IHC (Mab 3F52). Remarkably, in contrast to relatively comparable levels of *GLP1R* mRNA in EAT vs. SAT, GLP-1R immunopositive cells were much more abundant in EAT, compared to SAT (201). Tyramide signal amplification (which also has the potential to amplify signal from nonspecific primary antibody binding) was used to detect GLP-1R immunopositivity, due to the very low levels of GLP-1R expression, The precise identity of the GLP-1R+ cells in EAT was not determined. A follow-up study from the same group reported *GLP1R* expression in EAT by quantitative microarray analysis from 17 subjects undergoing coronary artery bypass grafting. Remarkably *GLP2R* mRNA transcripts were reported to be ~57-fold more abundant in EAT, relative to levels quantified for *GLP1R* (202).

Thyroid

GLP-1R agonists produce thyroid C-cell hyperplasia in rats and mice (203, 204), consistent with expression of the GLP-1R in calcitonin-producing C cells within the rat and mouse thyroid (203). The development of C cell proliferation and calcitonin secretion in response to GLP-1R agonism in mice and rats is mediated by the canonical GLP-1R (204, 205) prompting considerable regulatory concern, and more detailed investigation of GLP-1R expression in the human thyroid. Out of 59 normal, hyperplastic and neoplastic human thyroid glands analyzed by IHC with the Mab 3F52 antibody, no GLP-1R-immunopositive follicular or C cells were detected in normal or hyperplastic thyroid tissue. Two of ten cases with sporadic medullary thyroid carcinoma (MTC) exhibited scattered GLP-1R-immunopositive C cells (51). Receptor autoradiography using ^{125}I-labeled GLP-1 (7-36) amide detected the presence of GLP-1Rs in a

human case of MTC. Several studies have reported more widespread GLP-1R expression in the normal and neoplastic thyroid (206, 207). However, these analyses used GLP-1R antibodies lacking specificity (18, 20, 43). The available data suggests that GLP-1R+ cells are generally rare or absent in the normal human thyroid, yet may be expressed in a subset of C cells in the context of C cell hyperplasia or medullary thyroid cancer.

Eyes

Diabetic complications such as retinopathy, neuropathy, and nephropathy heighten the interest in the presence of GLP-1R in the eyes, peripheral nerves, and kidney. Although GLP-1R was reported in human retinal pigment epithelia by both qPCR and by IHC, these analyses used the subsequently discontinued ab39072 antisera (208). Subsequent studies using ISH and IHC with the GLP-1R antibody Mab 3F52 did not detect GLP-1R in the majority of cells within the human retina, however <1% of the cells in the ganglion cell layer were found to be GLP-1R+ (209). Using the same antibody, and protocols, GLP-1R-immunopositive cells were detected in GLP-1R+ tissues including the pancreas, Brunner's glands, and kidney.

Liver

There is considerable interest in the investigational assessment of GLP-1RA for the treatment of non-alcoholic steatosis (NASH), based on preclinical and human studies demonstrating these agents reduce hepatic fat, inflammation and progression of fibrosis (210). Nevertheless, the mechanisms linking GLP-1R signaling to control of NAFLD and NASH remain elusive, as levels of hepatic *Glp1r*/*GLP1R* expression are relatively low and sometimes undetectable,

relative to expression in pancreas, kidney, gut, lung, and heart (10, 211). Indeed, Boland and colleagues did not detect *GLP1R* mRNA transcripts in human liver, Kupffer or stellate cell fractions, prepared from 3 human liver biopsy samples analyzed by bulk RNA-seq (212). Although a substantial number of studies report hepatocyte GLP-1R expression in mouse and human liver by immunocytochemistry or Western blotting, the great majority of these analyses use antisera that have not been validated for sensitive and specific detection of the GLP-1R (191). Moreover, isolated murine hepatocytes do not exhibit canonical cAMP responses to GLP-1R agonists, fail to bind fluorescent GLP-1 in vitro, and do not express the *Glp1r* (19-21, 213).

GLP1R mRNA transcripts have been detected by RT-PCR in RNA isolated from human liver, and levels were reported to be reduced in livers from individuals with NASH (214). However, assignment of GLP-1R to hepatocytes was carried out using an antibody with unknown sensitivity and specificity. It seems likely that low level GLP-1R expression in liver reflects the presence of the GLP-1R in non-hepatocyte cells, such as immune cells or blood vessels. Indeed a fraction of ECs from mouse liver contained *Glp1r* mRNA transcripts detected by scRNA-seq (**Figure 5**) (155). Hence, it seems reasonable to conclude that the actions of GLP-1R agonists on hepatocytes are likely indirect, through incompletely identified pathways.

Concluding remarks for GLP-1 action outside the pancreas and nervous system

The availability data highlights multiple actions of GLP-1 and GLP-1R agonists, deduced from both preclinical and human studies, on key target cell types and tissues that do not exhibit robust GLP-1R expression. For example, GLP-1R agonists reduce the rates of myocardial infarction, decrease hepatic lipid accumulation, and attenuate renal albumin excretion, yet the GLP-1R is not expressed within most cardiomyocytes, hepatocytes, or renal glomerular and

tubular epithelium. Similarly, GLP-1R agonism leads to reduced atherosclerosis, attenuated chylomicron secretion and decreased inflammation, yet the majority of VSMs, ECs, enterocytes and immune cells do not express the canonical GLP-1R. Collectively, these findings highlight the importance of indirect mediators of GLP-1 action, ranging from improvement of metabolic control, weight loss, and acute neural or hormonal effectors, as key contributors to actions ensuing from activation of the canonical GLP-1R.

Caveats and limitations

Although considerable progress has been made in detecting GLP-1R expression, including characterization of two monoclonal antibodies suitable for detection of the primate and mouse/rat GLP-1R (3F52 and 7F38, respectively), it is still possible to incorrectly assign GLP-1R expression using these two antibodies. Importantly, the presence or absence of GLP-1R-immunopositivity remains highly technique- and tissue-dependent. Ideally, GLP-1R antisera should be regularly assessed for specificity under the conditions deployed for histochemistry or Western blotting using tissues from *Glp1r*$^{-/-}$ mice, or with human cells or organoids engineered to delete the GLP-1R. Although a predominantly membrane pattern of cellular staining is ideally observed when using chemical probes or validated antisera to detect the GLP-1R (18, 44, 49), several reports of diffuse cytoplasmic staining raise questions about whether the antisera were used under conditions optimized for specificity. For example, the 7F38 antisera detected diffuse cytoplasmic and some membrane GLP-1R-immunopositivity throughout the entire islet (158), a pattern of staining which can be more difficult to interpret, relative to the more restrictive cellular localization of islet and membrane-localized GLP-1R expression. Indeed, in the context

of establishing optimal technical conditions for the sensitive and specific detection of the human GLP-1R in human heart using IHC and the 3F52 antibody (18), we detected extensive cytoplasmic staining in tissues or cell types not known to express the GLP-1R. Hence, wherever possible, multiple complementary techniques should be used to interrogate the expression of the GLP-1R in specific cell types and tissues (18, 44, 49, 116, 118) including the use of negative controls and GLP-1R knockout tissues where applicable.

Many of the studies described herein utilize mice and rats as models for GLP-1 action and GLP-1R activation. The experimental conclusions may reflect the age, sex, species, housing conditions, diet, and health of the animals, as well as the specific experimental and technical approaches utilized. For example, the use of chemogenetic and optogenetic studies to interrogate PPG neurons informs the potential of broadly activating these neurons, but may not necessarily reflect the selective actions of GLP-1, acting through the GLP-1R, in the nervous system. Moreover, careful controls are required when using both these techniques, since optogenetic channels alter neuronal properties (215), and DREADD ligands such as clozapine-N-oxide can be converted to functional metabolites in the brain (216). Similarly, activation or deletion of the *Gcg* gene perturbs the expression of not only GLP-1, but multiple structurally related PGDPS, including oxyntomodulin and glucagon which may also signal through the GLP-1R. Hence, attribution of the results of these studies to the GLP-1/GLP-1R system should be done with caution. Furthermore, most studies localizing GLP-1 or the GLP-1R utilize post-mortem tissue and do not reveal information on GLP-1/GLP-1R expression in the live animal, or in live tissue.

This review did not examine the potential actions of GLP-1 degradation products, generated by enzymatic cleavage of GLP-1, which may produce multiple biological actions independent of the known GLP-1R (217). This biology may be relevant to circumstances characterized by pharmacological infusion of GLP-1, bariatric surgery, or GLP-1-producing tumors, and has been discussed elsewhere (6, 191). Moreover, although human islet GLP-1 action and GLP-1R expression has been extensively studied in the pancreas, much less is known about GLP-1R localization in other human organs and their cell types (Figure 8). As noted herein, the very low expression of the GLP-1R may preclude its reliable detection by RNA-seq, and more particularly in sc-RNA-seq, even in islet cells known to express the canonical GLP-1R and often used as a positive control (36). Hence, the old adage “absence of evidence is not evidence for absence” remains relevant for interpretation of these single cell or bulk data sets in tissues known to express low levels of the full length *GLP1R* mRNA. Along these lines, sensitive in situ transcript visualization techniques such as RNAScope and single molecular fluorescence ISH might prove transformative for understating GLP-1R mRNA localization and abundance in complex tissues.

Recent technological advances coupled with reagent development have fostered a more accurate and complete understanding of how and where endogenous and pharmacologically administered GLP-1 and GLP-1RA acts to control metabolism. Rapid ongoing developments in this field are likely to further refine existing insights, answering important mechanistic questions, while raising new testable hypotheses designed to illuminate the expanding direct vs. indirect cellular targets of GLP-1 action.

Abbreviations

αSMA=α-smooth muscle actin

AP=Area Postrema

ARC=Arcuate nucleus

bp=base pair

CCK=Cholecystokinin

cDNA=Complementary deoxyribonucleic acid

cAMP=cyclic adenosine 3',5'-monophosphate

CNS=Central Nervous System

DPP-4=Dipeptidyl Peptidase-4

DTA=Diphtheria Toxin

DREADD= Designer Receptors Activated by Designer Drugs

EAT=Epicardial Adipose Tissue

EC=Endothelial cells

EC_{50}=Effective concentration half maximal

ENS=Enteric Nervous System

EPAC=Exchange protein directly activated by cAMP

ER=Endoplasmic Reticulum

Ex4=Exendin-4

Ex9=Exendin(9-39)

FACS=Fluorescence-Activated Cell Sorting

GABA=γ-aminobutyric acid

GAD-Glutamic Acid Decarboxylase

GCG=Glucagon gene or protein

GFP=Green Fluorescent Protein

GLP-1=Glucagon-like peptide-1

GLP-1R=Glucagon-like peptide-1 receptor

GLP-1RA= Glucagon-like peptide-1 receptor agonists

HFD=High Fat Diet

ICV=Intracerebroventricular

IEL=Intestinal intraepithelial lymphocyte

IHC=Immunohistochemistry

iNKT cells=Invariant Natural Killer T cells

ISH=In situ hybridization

LA=Left Atrium

LV=Left Ventricle

Mab=Monoclonal Antibody

Median Eminence

MHC=Myosin Heavy Chain

MTC=Medullary Thyroid Cancer

NG2=Neural/glial Antigen 2

NG=Nodose Ganglion

NTS=Nucleus Tractus Solitarius

OVLT=Organum vasculosum of the lamina terminalis

PGDPs=Proglucagon-derived peptides

PKC=Protein kinase C

PLC=Phospholipase C

PPG=Preproglucagon

qPCR=quantitative PCR

RA=Right Atrium

RV=Right Ventricle

RNA-seq=Ribonucleic acid sequencing

scRNA-seq= Single cell Ribonucleic acid sequencing

RT-PCR=Reverse Transcription-Polymerase Chain Reaction

SiR=Silicon rhodamine

SFO=Subfornical organ

SSTR=Somatostatin Receptor

SSTR2=Somatostatin Receptor 2

SDA=Subdiaphragmatic vagal afferent deafferentation

SAT=Subcutaneous Adipose Tissue

TCR=T Cell Receptor

TMR=Tetramethylrhodamine

T2D=Type 2 Diabetes

vGAT=vesicular GABA Transporter

vGLUT2= Vesicular glutamate transporter 2

VSM=Vascular smooth muscle cells

YFP=Yellow Fluorescent Protein

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Figure Legends

Figure 1: GLP-1 source, GLP-1R labeling and GLP-1R signalling in pancreatic islets

A) Potential sources of GLP1 in the islets, as well as paracrine signaling pathways. Proglucagon products from α-cells that activate islet GLP-1Rs include glucagon and potentially GLP-1. The biological relevance of GLP-1 produced by α-cells remains an unresolved line of investigation.

B) GLP1R labelling is observed at the membrane in β-cells. Predominantly cytoplasmic labelling is non-specific unless the GLP1R is agonist-bound in which case some surface and punctate intracellular staining is detected due to GLP1R internalization.

C) Major GLP1R signaling pathways reported in primary rodent and human beta cells or islets. It is well established that GLP-1R signals through cAMP pathways, including PKA and EPAC2. β-arrestin 1 has been proposed to regulate GLP-1R signaling, however, the mechanisms of this pathway remain unresolved.

D) Actions mediated by the GLP-1R in pancreatic islets.

ADCY, adenylate cyclase, GLP1R, Glucagon-like peptide-1 receptor; PKC, protein kinase A; EPAC2, exchange protein directly activated by cAMP 2; INS, insulin SST, somatostatin.

Figure 2

The UMAP (Uniform Manifold Approximation and Projection) plots were created to visualize gene expression in each cell. The x-axis and y-axis indicate the overall transcriptional difference of each cells arbitrated as “distance” in a two dimensional space; the closer the two cells on the plot, the more similar their transcriptomes are, and in turn they are more likely to share a lineage.

The expression scales are continual and log2 normalized, ranging from light blue (low expression) to dark red (high expression) in colour, Cell types were classified based on markers genes *INS* and *MAFA* (mature β cells), *GCG* (α cells), *SST* (δ cells), and *PPY* (PP cells). Count matrices were accessed on 19th Sep 2020 and aggregated from the scRNA-seq datasets reported by Segerstolpe et al. (37) (GEO accession number GSE73727), Enge et al. (218) (GEO accession number GSE81547), and Camunas-Soler et al. (38) (GEO accession number GSE124742). Human gene nomenclature: *INS*, insulin; *GCG*, glucagon; *SST*, somatostatin; *PPY*, pancreatic polypeptide Y; *MAFA*, V-maf musculoaponeurotic fibrosarcoma oncogene homologue A.

Figure 3

GLP-1 receptors (GLP-1Rs) and brain-derived GLP-1 in the central nervous system. Within the CNS GLP-1 is produced by PPG neurons (green areas) and distributed to their axon terminals ready for synaptic release (dark green). Names of brain areas that receive PPG innervation and express GLP-1 receptors are given as abbreviations in blue. Systemically distributed GLP-1 receptor agonists (GLP-1RAs) are depicted as red circles and access the circumventricular organs with a leaky blood-brain barrier, and the ventricles, but not the brain parenchyma. The olfactory bulb and the piriform cortex (Pir) form areas that express GLP-1Rs and harbour PPG neurons that project locally only. Abbreviations: AH: anterior hypothalamus; AP: area postrema; ARC: arcuate nucleus; Barr: Barrington's nucleus; BNST: bed nucleus of the stria terminalis; CAA: central automonic area (lamina X); caud Hipp: caudal ventral hippocampus; CeA: central nucleus of the amygdala; DMH: dorsomedial hypothalamus; DMNX: dorsal vagal motornucleus; GrO: granule cell layer of the olfactory bulb; IML: intermediolateral nucleus; IRT: intermediate reticular nucleus; LC; locus coeruleus; NAc: nucleus accumbens; OVLT: organum vasculosum of the lamina terminalis; PAG:

periaqueductal grey; PBN: parabrachial nucleus; Pir: piriform cortex; PVN: paraventricular nucleus; PVT: paraventricular thalamus; Sep: lateral septum; SFO: subfornical organ; RPa: raphe pallidus; VLM: ventrolateral medulla; VMPO: ventromedial posterior nucleus.

Figure 4

GLP-1 receptor expression in heart and blood vessels within select organs and actions of GLP-1 associated with these organ and cell types. The arrow depicts the relative levels of GLP-1R expression deduced from human gene expression databases such as the Genotype Tissue Expression portal

Figure 5

Expression of mouse *Glp1r* in 40,449 murine endothelial cells. Count matrices were accessed on 19th Sep 2020 and reported by Kalucka et al. (219) (ArrayExpress ID E-MTAB-8077).

Figure 6

Co-expression of the proglucagon gene (*Gcg)* and enteroendocrine genes in 411 murine small intestine L cells. Count matrices were accessed on 19th Sep 2020 and retrieved from the scRNA-seq datasets reported by Gehart *et al* (167) (GEO accession number GSE113561). Mouse gene nomenclature: *Pyy*, Peptide YY; *Gip*, glucose-dependent insulinotropic polypeptide; *Nts*, Neurotensin; *Tph1*, Tryptophan hydroxylase 1;*Sst*, Somatostatin; *Ghrl*, Ghrelin; *Sct*, Secretin; *Cck*, Cholecystokinin.

Figure 7

Expression of *Glp1r* in 4,974 murine ileal and colonic enteric neurons. Enteric neuron subtypes types were classified based on neuropeptide genes *Adcyap1* (PACAP neurons and *Gal* (galanin neurons). Count matrices were accessed on 19th Sep 2020 and aggregated from the scRNA-seq datasets reported by Drokhlyansky et al (183) (Broad Institute Single Cell Portal

SCP1038). Mouse gene nomenclature: *Adcyap1*, pituitary adenylate cyclase activating polypeptide or PACAP; *Gal*, galanin.

Figure 8

Schematic depiction of major targets of GLP-1 action, and the cell types within key organs that express the GLP-1 Receptor (GLP1R). The GLP-1 peptide is produced in and shown adjacent to the intestine, pancreas, and brain, the three major sites of *GCG* expression. EC=Endothelial Cells; IEL=Intestinal Intraepithelial Lymphocytes; SA node=Sinoatrial node; VSMC=Vascular Smooth Muscle Cell.

Cell type	*Glp1r/GLP1R*	GLP1R
β-cell	+++; robust and ubiquitous expression	+++; robust and ubiquitous expression, established direct GLP-1 activity
α-cell	+/-; little to no expression	+/-; little to no expression, direct GLP-1 activity is debated
δ-cell	++; ubiquitous, but lower expression relative to β-cells	+/-; little or no expression, evidence for direct GLP-1 activity is minimal

Table 1: relative GLP1R transcript and protein levels in the islet endocrine cell types based upon (33-36, 40, 41, 46, 48, 49)

Reagent type	Catalogue number/name	Source	Reported cross-reactivity	Reference
Antibody	Mab 3F52	Iowa DSHB	Human, primate	(44)
Antibody	Mab 7F38	Iowa DSHB	Human, Mouse, Rabbit, Rat	(119)
Antibody	Glp1R0017/ GLP1R-APC	Cambridge University	Mouse	(36, 53)
GLP1R agonist	Ex4-Cy3 and Ex4-Cy5	Novo Nordisk	Mouse	(48)
GLP1R agonist	Liraglutide^{750}	Novo Nordisk	Mouse, rat	(118)
GLP1R antagonist	Ex9-39^{750}	Novo Nordisk	Mouse, rat	(118)
GLP1R antagonist	LUXendin555, LUXendin645 and LUXendin651	University of Birmingham	Mouse/hESC	(49)

Table 2: List of antibodies and fluorescent ligands validated for GLP1R detection specificity using GLP1R$^{-/-}$ tissue or transfected cells (with and without human GLP1R).