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

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44 any of these companies.

45 **Essential Points**

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

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

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

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

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

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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.

74 **Introduction**

75

76 The gut endocrine system contains dozens of specialized plurihormonal endocrine cells whose
77 peptide hormone products regulate a wide range of biological functions, ranging from control of
78 ingestive behavior to the digestion, absorption, and assimilation of nutrients (1). Among the
79 best studied of the gut hormones is glucagon-like peptide-1 (GLP-1), a product of the
80 proglucagon gene (*GCG*). The cloning of the proglucagon cDNAs encoding GLP-1 within the
81 proglucagon (*GCG*) gene in the early 1980s (2, 3), enabled the study of the physiology of the
82 glucagon-like peptides. Originally described as an incretin hormone that potentiates meal-
83 stimulated insulin secretion, several decades of research have broadened the actions of GLP-
84 1 beyond the endocrine pancreas to encompass activity within the nervous system, gut, kidney,
85 pancreas, heart, and immune system (4-6). Moreover, the clinical development of GLP-1R
86 agonists (GLP-1RA) for the treatment of type 2 diabetes (T2D) and obesity has heightened the
87 interest in how both physiological and pharmacological levels of GLP-1 act on key tissues
88 relevant to the pathophysiology of diabetes and its complications.

89

90 A major advance in understanding GLP-1 action stemmed from the cloning and characterization
91 of the GLP-1 receptor (GLP-1R) (7). *GLP1R* mRNA transcripts were found to be widely
92 distributed in peripheral tissues and the central nervous system (CNS), providing a logical link
93 between the demonstrated actions of GLP-1 and the expression of the canonical GLP-1R in
94 cellular targets of GLP-1 action (8-10). Nevertheless, the precise cellular localization of GLP-
95 1R has proved to be challenging, reflecting low levels of mRNA expression, and multiple
96 technical challenges, including the inadvertent application of incompletely characterized non-
97 specific antisera for detection of GLP-1R protein expression (11).

98

99 The concept of GLP-1 as a gut-derived hormone acting on peripheral organs, has been refined
100 through enhanced understanding of the importance of pancreatic- and brain-derived GLP-1.

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

111

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

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

124

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

132

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

143

144 **The Pancreas**

145

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).

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

202

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

218

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

235

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

237

238 Multiple approaches have localized GLP-1R in pancreatic islets. Early studies using
239 immunohistochemistry (IHC) to examine rat pancreas showed immunoreactivity for GLP-1R in
240 the majority of β -cells and δ -cells, with 1 in 5 α -cells also expressing the receptor, in line with
241 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).

257

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 ($\sim 1\%$) (48). Along these lines, well characterized

266 fluorescent antagonists revealed predominant GLP-1R expression in β -cells (~85%), with
267 slightly higher expression in α -cells (~ 5%) than shown with fluorescent agonist or antibody
268 (49) but in agreement with *Glp1r-Cre:ROSA26-tdRFP:GLU-Venus* double reporter mouse
269 approaches (35). Notably, for all fluorescent-ligand localization approaches, staining
270 overlapped with data obtained using IHC with antibodies validated in *Glp1r^{-/-}* mouse tissue (48,
271 49).

272

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

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

280

281 Early studies looking at human islets showed strong co-localization of GLP-1R with β -cells, with
282 no staining seen in α -cells (41). Similarly, studies using a commercial antibody confirmed these
283 results, showing GLP-1R immunopositivity in 0.25% of α -cells (50). It is worth noting however
284 that the antibodies used in both these studies gave rise to predominantly cytoplasmic staining,
285 at odds with the known localization of non-stimulated GLP-1R. Studies using a validated
286 antibody with membrane-localized staining again showed GLP-1R expression largely confined
287 to β -cells, but staining was not performed for glucagon, making quantification impossible (44).
288 Consistent with these observations, Waser and colleagues utilized the Mab 3F52 antibody and
289 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

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

320

321

322 **Consensus view of islet GLP-1R expression**

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

338 subpopulations of cells. For instance, if less than 10% of α -cells are *Glp1r*⁺, the remaining
339 90+% of α -cells in the binned sample may dilute this signal to yield the interpretation that α -
340 cells are *Glp1r*⁻. Thus, combining multiple approaches to interrogate GLP-1R expression
341 remains necessary to account for the limitations of a single approach. Table 1 summarizes
342 GLP-1R transcript and protein levels based on the studies outlined above.

343

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

345

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

361

362 The differing concentrations of GLP-1 used in these experiments could reconcile the discrepant
363 results in the β -arrestin 1 knockout models, as high concentrations of GLP-1 (100 nM) (59) may
364 elicit different signaling pathways compared to GLP-1R activation with lower concentrations
365 (0.3 nM) (62). Moreover, the absence of a phenotype in the single β -arrestin knockout models
366 (59-61) could equally reflect the presence of redundancy in the GLP-1R signalling network and
367 as such studies in double β -arrestin 1/2 knockout mice are needed. The major GLP-1R
368 signaling pathways in rodents and human β -cells are shown in **Figure 1C**.

369

370 Activation of GLP-1R by picomolar concentrations of GLP-1 has been demonstrated in mouse
371 and human β -cells (63, 64). Rather than engaging cAMP synthesis, low concentrations of GLP-
372 1 instead activate a phospholipase C (PLC)-protein kinase C (PKC)-Transient Receptor
373 Potential Cation Channel Subfamily M Member (TRPM)4/5-signalling cascade through the Gq α
374 subunit, stimulating insulin secretion (63). Low concentrations of GLP-1 also to increase Ca²⁺
375 fluxes in human β -cells, which fits with a role for Inositol 1,4,5-trisphosphate 3(IP3)-dependent
376 mobilization of endoplasmic reticulum (ER) Ca²⁺ stores (IP3 being downstream of PKC) (63,
377 64). Most recently, GLP-1R has been shown to signal predominantly via Gq α in mouse and
378 human β -cells subject to chronic depolarizing stimuli (K_{ATP} channel mutations, hyperglycemia)
379 (65). Since several of these experiments used chemical inhibitors with known off-target effects
380 (e.g. on store-operated Ca²⁺ entry, phosphodiesterases), it will be important to examine
381 whether similar phenotypes exist in β -cells specifically deleted or knocked down for Gq α or Gs α .
382 Lastly, GLP-1 also engages a network of β -cells in human islets, leading to coordinated Ca²⁺
383 rises (64). Such signalling is dependent on intercellular gap junction communication, which Ex4
384 has been shown to facilitate in a cAMP-protein kinase A (PKA)-EPAC-dependent manner (66).

385

386 α -cells: The hypothesis that GLP-1 directly affects α -cell function remains controversial. Despite
387 evidence supporting GLP-1R expression in only a small subpopulation of α -cells, studies have
388 shown that GLP-1 influences α -cell signalling and glucagon secretion by inhibiting N and P/Q
389 voltage-dependent Ca^{2+} channels (42, 45, 50). α -cells could plausibly express low levels of
390 GLP1-R, which would be difficult to detect using immunostaining, yet sufficient to evoke a
391 response in a highly-amplified signalling cascade. However, low abundance *Glp1r* mRNA
392 transcripts should still lead to recombination in the various GLP-1R-reporter mice discussed
393 above, where somewhere between 0% (36) and 10% (35) of α -cells were labelled with reporter.
394 Moreover, the most recent fluorophore antagonist approaches have single-molecule resolution
395 and detected GLP-1R in only 1-5% of α -cells, with either complete absence or strong signal,
396 suggesting that graded expression in the population is unlikely (49). Another explanation for
397 the actions of GLP-1 on α -cells could be through GLP-1 degradation products. Several reports
398 suggest that GLP-1(7-36) and GLP-1(9-36) are glucagonostatic in wild-type and *Glp1r*^{-/-} islets
399 (67). Effects of GLP-1(7-36) but not GLP-1(9-36) were abolished by pre-treatment with a DPP-
400 4 inhibitor, suggesting that GLP-1(7-36) is pro-peptide for GLP-1(9-36) in this setting (67).
401 Notably, GLP-1(9-36) was found to signal at the human glucagon receptor with an EC_{50} = 30
402 nM. While such concentrations are pharmacological, GLP-1(9-36) is the major circulating form
403 of GLP-1 and DPP4 is present in islets, which could plausibly generate high local levels of
404 cleavage products. These findings are however difficult to reconcile with previous studies
405 showing that the GLP-1R-specific antagonist Ex9 (27) blocks the effects of GLP-1 on α -cell
406 electrical activity and glucagon secretion (42). Although interesting, these data are unlikely to
407 be relevant for degradation-resistant GLP-1RA and DPP-4 inhibitors, which suppress glucagon
408 secretion without generating GLP-1(9-36) through mechanisms requiring the known GLP-1R.
409

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

423

424 ***GLP-1R expression and function in the exocrine pancreas***

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

434 with or without diabetes (44). Similar patterns of acinar cell GLP-1R-immunopositivity were
435 detected in pancreatic specimens obtained at surgery from 10 non-diabetic individuals, as well
436 as in 12 pancreatic specimens from individuals with T2D. In situ ligand binding detected GLP-
437 1 binding sites in the pancreatic acinar cells, with radiolabelled ligand displaced in the presence
438 of excess unlabelled GLP-1 (44). Waser and colleagues examined pancreatic GLP-R
439 expression in the human pancreas by IHC using the Mab 3F52 antibody, as well as by in situ
440 ligand binding autoradiography using ¹²⁵I- GLP1(7–36)-amide (51). A combination of both
441 diffuse and heterogeneous weak GLP-1R expression was observed in some but not all acini
442 from pancreatic samples resected from insulinoma tumors, chronic pancreatitis and a few cases
443 of pancreatic cancer. No immunoreactive GLP-1R expression was detected in pancreatic ducts
444 (51). Similar patterns of acinar cell GLP-1R localization were reported in the mouse pancreas,
445 using the ¹²⁵I-labeled GLP-1R antagonist Ex9 to detect in situ binding, as well as the Mab 7F38
446 antibody to localize GLP-1R immunopositivity in the exocrine pancreas of WT but not *Glp1r*^{-/-}
447 mice (73). Hence, the available data supports low level GLP-1R expression in the pancreatic
448 acinar but not ductal cells in mice and humans.

449

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

451 Actions mediated by GLP-1 in the islet are shown in **Figure 1D**. The major source of GLP-1
452 that binds GLP-1R in the healthy islet is still unresolved, although GLP-1 produced in α -cells
453 may play a relatively more important role in the injured pancreas. Insulinotropic effects of GLP-
454 1 and GLP1-RA are mediated by GLP-1Rs expressed predominantly (if not completely) in the
455 β -cell compartment in both mice and human islets, assessed using validated antibodies, probes
456 and reporter mice across a range of techniques. Direct effects of GLP-1 on α -cell function and
457 glucagon secretion remain debated and are unlikely to be mediated by the GLP-1R. Instead,

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

464

465 **The Brain GLP-1 system**

466 ***Where does brain GLP-1 originate from?***

467 Proglucagon (GCG) mRNA transcripts are detected predominantly in the brainstem (74, 75),
468 whereas the glucagon-like peptides (including GLP-1), and the GLP-1R are more widely
469 distributed throughout the central nervous system (CNS) (10, 75-80). Further characterization
470 of the GLP-1-immunoreactive cells in the lower brainstem solidified the view that these cells
471 are a specific population of neurons, rather than glial cells or endothelial cells (ECs), that are
472 not catecholaminergic but primarily glutamatergic (79, 81). Additionally, the observation that
473 GLP-1 immunoreactivity was localised to axon terminals in the hypothalamus further solidified
474 the view that GLP-1 -cells in the brainstem are neurons and utilise GLP-1 as a neurotransmitter
475 that is stored until release in the axon terminals. A first proof that this assumption is correct was
476 provided by the observation that injection of a virus encoding shRNA against PPG into the NTS
477 causes a significant decrease in GLP-1 immunoreactivity in the paraventricular nucleus of the
478 hypothalamus (82). Functional studies in the rat combining immunohistochemical identification
479 of these neurons with c-Fos immunodetection revealed their activation by malaise, stress and
480 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**

505 allowed the selective ablation of NTS-PPG neurons and thereby the demonstration that loss of
506 these neurons substantially decreased GLP-1 content in both hypothalamus and spinal cord
507 (100) and thus corroborated the findings from rat, that interference with GLP-1 translation in the
508 brainstem affects GLP-1 content in the forebrain (82). The 'second generation' *Gcg-Cre*
509 transgenic mice additionally provided insights where Cre-dependent viral expression of
510 Channelrhodopsin2-Green Fluorescent Protein (ChR2-GFP) was used to confirm the
511 assumption that NTS-PPG neurons project to most (if not all) forebrain targets (103). These
512 findings confirmed results of prior studies utilizing injection of retrograde tracers into various
513 forebrain areas and co-localizing the tracer with GLP-1 immunoreactivity in the NTS and IRT
514 (79, 104).

515 ***Manipulation of PPG neurons to interrogate their function in vivo***

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

523 This was followed by a study employing both inhibitory DREADD receptor expression as well
524 as selective ablation of NTS PPG neurons by the expression of diphtheria toxin A subunit (DTA)
525 to examine effects on food intake and brain GLP-1 content (100). Both hypothalamic and spinal
526 cord GLP-1 content was severely reduced upon ablation of these neurons, thus demonstrating
527 that brainstem GLP-1 neurons are the principal source of GLP-1 within the CNS and that GLP-
528 1 is stored in the axon terminals of the PPG neurons, all of which have their cell bodies in the

529 lower brainstem. Interestingly, as observed before with shRNA knockdown in rat, there was
530 little effect on basal food intake, but reduction of brainstem *Gcg* expression or ablation of PPG
531 neurons impaired control of post fasting food intake and stress induced feeding reduction (82,
532 100).

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

543 ICV injection of the GLP-1R antagonist Ex9 in chow-fed rats and mice impaired glucose
544 tolerance (82, 105), but ablation of PPG neurons in chow-fed mice did not impair glucose
545 tolerance (100). The simplest explanation for this discrepancy would be that PPG neurons are
546 not involved in the regulation of glucose tolerance, but that gut-derived GLP-1 is able to reach
547 (parts of) the brain to affect glucose tolerance, or that ICV Ex9 actually accesses the general
548 circulation and exerts direct effects, e.g. in the pancreas. Alternatively, basal constitutive GLP-
549 1R signaling may exert tonic control of these processes, which may be disrupted by
550 pharmacological administration of GLP-1R antagonists independent of the source of the GLP-
551 1 ligand (106-108). Finally, chemogenetic activation of PPG neurons increases heart rate, but
552 their inhibition or ablation has no effect on resting or active heart rate (HR) or blood pressure

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

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

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

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

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

588 **GLP-1 receptors within the nervous system**

589

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

593 Complementary studies have used mice with germline or conditional tissue-specific deletion of
594 the GLP-1R to examine the importance of different GLP-1R populations in the CNS and
595 periphery (121-126). Notably, the hypophagia caused by ICV injection of GLP-1 was fully
596 abrogated in *Glp1r*^{-/-} mice, consistent with the loss of binding sites for Ex4 in the *Glp1r*^{-/-} mouse
597 brain (121). These findings align with the demonstration that PPG neuron activation produces
598 hypophagia, but that the loss or attenuation of PPG neuronal activity or genetic deletion of gut
599 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

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

650 *GLP-1Rs in circumventricular organs*

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

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

672 The available evidence distinguishes between the weight lowering effects of systemically
673 administered GLP-1RA from those of PPG neuronal activation. This notion is supported by
674 studies dissociating the hypophagic effects of PPG neuron activation from the **food intake**
675 **suppressing** actions of subcutaneous semaglutide (135). It seems possible that GLP-1Rs
676 expressed in the ARC may be activated by high circulating levels of GLP-1 under conditions of
677 severe intestinal malaise or following bariatric surgery. This distinction may explain why GLP-
678 1RA therapy is associated with an increased sensation of nausea (136), whilst chemogenetic
679 activation of brainstem PPG neurons does not elicit adverse responses in mice (102, 135).

680 ***GLP-1Rs inside the blood-brain barrier***

681 Most other GLP-1R-expressing brain regions receive axonal inputs from PPG neurons, and are
682 likely activated by GLP-1 originating from those cells. However, brainstem PPG neurons do not
683 project to the hippocampus or to the cerebral cortex (93). Thus, it is currently unclear how GLP-
684 1Rs located in these areas are activated. A small population of PPG neurons has been reported
685 in the piriform cortex (93), but these do not give rise to axons in other parts of the cortex or the
686 hippocampus. Activation of GLP-1Rs in the ventral hippocampus also results in hypophagia,
687 and it has been suggested that volume transmission from the lateral ventricles, which had
688 detectable levels of GLP-1, might account for their physiological activation (137). These
689 observations are consistent with the possibility that PPG neurons might be the source of GLP-
690 1 within the ventricle, as PPG axons are found along the ependymal cell layer covering the
691 surface of the ventricles and intraventricular injection of a retrograde tracer labelled a fraction
692 of NTS PPG neurons (137). Whilst this is plausible, it should be noted, that both GLP-1RA and
693 GLP-1R antagonists given subcutaneously access the ventricular space (49, 116, 118). Hence,
694 there is no discernable barrier for pharmacological or gut-derived GLP-1 to reach the brain
695 ventricles, but it does not provide evidence that post-prandially released GLP-1 would

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

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

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

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

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

728 **GLP-1R expression in the cardiovascular system**

729 Cardiovascular safety trials with GLP-1RA have demonstrated reduction in all cause- and
730 cardiovascular mortality, and lower rates of major cardiovascular events including stroke and
731 myocardial infarction (15, 16). GLP-1RA also increase HR in humans and animals, findings
732 attributed in part to activation of the sympathetic nervous system, inhibition of the
733 parasympathetic nervous system, and activation of the sinoatrial GLP-1R (143, 144, 147, 148).
734 GLP-1R signaling also attenuates the extent of experimental stroke in preclinical studies (97),
735 and GLP-1RA reduce the rates of stroke in humans with risk factors for or established
736 cardiovascular disease (149). Furthermore, GLP-1RA also reduce albumin excretion, and trials
737 are underway to determine whether these agents may reduce rates of dialysis, decline of eGFR
738 and renal replacement, in people with diabetic kidney disease. Collectively, these observations
739 have created great interest in understanding how GLP-1RA exert their mechanisms of action
740 in the cardiorenal system. Nevertheless, there is still relative uncertainty and some controversy
741 about the precise cell types and tissues that express the GLP-1R in the heart, peripheral
742 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, ¹²⁵I-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

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

769

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

783

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

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

800

801 **Assessment of GLP-1R expression in blood vessels**

802

803 Despite the multiplicity of studies describing actions of GLP-1RA on blood vessels, there is
804 surprisingly little rigorous information that describes GLP-1R expression in normal or diseased
805 VSMs, ECs or other vascular cell types. Much of the data in this area relies on ECs and VSMs
806 analyzed after cell culture ex vivo, which may not be representative of GLP-1R expression in
807 the same cell types in vivo. Indeed *GLP1R* mRNA transcripts were not detected by qPCR in
808 RNA isolated from human coronary artery VSMs or ECs (18). However, Richards reported
809 *Glp1r* promoter-directed fluorescent cells in the aorta, and arteries and arterioles of multiple
810 tissues, and *Glp1r* mRNA transcripts were detected by qPCR in mouse aorta, albeit at levels
811 90% lower than corresponding expression of *Glp1r* in RNA isolated from mouse atrial tissue
812 (35). Fluorescent cells within the intestinal vasculature co-stained with α -smooth muscle actin
813 (α SMA) and Neural/glial antigen 2(NG2), a marker for pericytes, and were thought to represent

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

816

817 *GLP1R* mRNA transcripts were detected by qPCR in the aortic intima and media obtained from
818 subjects, with and without obesity, scheduled for aortic surgery (154). Extensive diffuse GLP-
819 1R-immunopositivity was detected in the aorta using Mab 3F52. Notably, *GLP1R*/GLP-1R
820 expression was not quantified relative to other *GLP1R*+ tissues, nor assessed using the same
821 reagents and conditions for comparative purposes of specificity in organs known not to express
822 the GLP-1R.

823

824 Single cell analysis of murine endothelial cells from 20 organs showed *Glp1r*+ cells most
825 prominently in the lung (**Figure 5**), with some positive ECs also detected in cells from the liver
826 and heart (155). Given the limitations of read depth needed to identify low abundance
827 transcripts with scRNA-seq, this is likely a conservative snapshot of EC *Glp1r* expression.
828 Consistent with *Glp1r* expression in ECs, mice expressing EC-specific *Cdh5*-dependent Cre
829 recombinase exhibited reduced *Glp1r* expression in a lung EC preparation isolated using CD31
830 and CD102 coated beads (156). However, no markers were used to assess the extent of EC
831 enrichment, and knockdown of *Glp1r* was not examined in resistance arteries, kidneys, heart,
832 aorta or other vascular sites that might contain EC GLP-1R expression. Using *Glp1r*-Cre mice
833 crossed with a ROSA26-tdRFP reporter, Richards and colleagues also detected *Glp1r*-directed
834 reporter gene expression in a few blood vessels within the cardiac ventricles, with a morphology
835 consistent with VSMs (35). Overall, the data suggests that *Glp1r*/*GLP1R* expression in the
836 vascular system is highly heterogeneous, with high expression in mouse lung ECs and
837 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 ¹²⁵I-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

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

867
868 **GLP-1 production in enteroendocrine L cells along the gut**

869
870 Enteroendocrine L cells have classically been defined as the cell type giving rise to GLP-1 in
871 the gut (1). In humans and mice, the majority of L cells reside in the ileum and colon, with fewer
872 cells located in the duodenum and jejunum. L cells sense various nutrients from the luminal
873 surface and in turn secrete GLP-1 into the local circulation (160). Numerous cognate receptors
874 for non-nutrient GLP-1 secretagogues have also been identified on the basolateral side of L
875 cells, thus enabling L cells to simultaneously sense molecules in the circulation (161-163). L
876 cells in the proximal gut are capable of responding briskly to nutritive stimuli (14, 164); the
877 available data suggests that distal gut L cells exhibit secretory responses biased towards non-
878 nutrient secretagogues (164). Analysis of mice with marked reduction of *Gcg* expression within
879 the entire, or distal gut, reveals essential contributions of intestinal *Gcg* expression to the pool
880 of circulating GLP-1, and in the control of gastric emptying and glucose homeostasis (14).

881
882 scRNA-seq analyses have illuminated the transcriptomic heterogeneity evident across subsets
883 of L cells (**Figure 6**). One of the first scRNA-seq studies on the mouse small intestine recovered
884 310 out of 7216 (4.3%; which is higher than often achieved) quality control-passed EECs (165),
885 and 10% of these were identified as *Gcg*⁺. Subsequently, several reports characterized

individual L cells isolated from mouse reporter lines. Sequencing of 259 individual YFP⁺ cells isolated from the small intestine of GLU-Venus mice identified three main subgroups of L cells. These corresponded to 1) *Gcg/Pyy* double-positive cells, which account for 50% of L cells; 2) *Gcg/Tph1/Pzp* triple-positive cells, which account for 35% L cells; and 3) *Gcg/Gip* double-positive cells, which account for 15% L cells (166). Similarly, a comprehensive survey of 6,906 small intestine EECs from *Neurog3Chrono* reporter mice identified more than 90% of L cells as multi-hormonal (167). The detection of *Gcg* in EECs correlated strongly with *Cck*, followed by *Pyy*, *Ghrl*, and *Sct* co-expression (**Figure 6**). A scRNA-seq study on 1,560 intestinal YFP⁺ cells isolated from *Neurod1-Cre; Rosa26-YFP* mice confirmed that colonic L cells are also mostly multi-hormonal (168). Although both small and large bowel L cells can produce neurotensin, L cells in the distal colon preferentially expressed *Pyy* and *InsI5*. Studies of human EECs have relied primarily on bulk RNA-seq for transcriptome profiling (169-171). To circumvent the difficulty of isolating L cells from human intestine with high purity, a recent study sequenced 2,255 cells single cells from human small intestinal organoids transduced with doxycycline-inducible *NEUROG3* transgenes to drive differentiation towards endocrine lineages (172). Three L cell subtypes among the 2,255 EECs sequenced were *PYY*^{High}, *PYY*^{Low}, and *NTS*^{High} cells, all of which were also detected in the mouse L cell scRNA-seq datasets. A discrepancy between the mouse and human data was the absence of *TPH1*⁺ and *GIP*⁺ L cells, although the number of organoid-derived L cells sequenced in the study was not high (< 100), and the L cell subtypes in the organoid might not faithfully recapitulate the ones in the human intestine *in situ*. Nevertheless, the analyses and corroboration of mouse and human L cell scRNA-seq datasets support a conserved transcriptional program in L cells across the species. Analysis of human jejunal L cell transcriptomics, and L cell density identified dysregulation of enteroendocrine differentiation signatures associated with reduced L cell density in obese people with T2D (173),

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

918

919 **GLP-1R expression in the gastrointestinal tract**

920

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

927

928 **GLP-1R expression and biology in gut intraepithelial lymphocytes**

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

934 augments cyclic adenosine monophosphate (cAMP) levels in murine gut IELs *ex vivo* (174).
935 Within the intestinal epithelium, both TCR $\gamma\delta^+$ and TCR $\alpha\beta^+$ IELs express comparable levels of
936 *Glp1r* as shown by qPCR on sorted IELs (175). The importance of gut IEL GLP-1Rs for
937 regulating incretin biology and metabolism has been examined in the context of $\beta 7^{-/-}$ mice,
938 which lack gut IELs due to defects in lymphocyte gut homing (175). Reconstitution of irradiated
939 *Ldlr*^{-/-} mice with a chimera of $\beta 7^{-/-}$ /*Glp1r*^{-/-} bone marrow raised plasma GLP-1, improved
940 intraperitoneal glucose tolerance, lowered plasma cholesterol, and reduced aortic plaque size
941 after 14 weeks of high-fat high-cholesterol diet feeding, relative to irradiated WT mice with a
942 chimera of $\beta 7^{-/-}$ /WT bone marrow. How gut GLP-1R-expressing IELs control the bioavailability
943 of local and systemic GLP-1 remains uncertain (175). The physiological importance of the
944 enteroendocrine-IEL axis for local and systemic immunity remains incompletely defined.

945

946 ***Stomach and Brunner's glands express the GLP1R***

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

953

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

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

960

961 ***GLP-1R expression in the enteric nervous system***

962

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

968

969 Multiple lines of evidence demonstrate GLP-1R expression in subsets of enteric neurons. A
970 study using mouse reporter lines expressing *Rosa26*-tdRFP or *Rosa26*-eYFP under the control
971 of *Glp1r*-Cre found a small number of fluorescent-positive enteric neurons (35); treatment with
972 10 nmol/L GLP-1 increased their action potential firing frequency *ex vivo*. About 60% and 19%
973 of these fluorescent-positive neurons from the small and large intestine, respectively, were
974 positive for neuronal nitric oxide synthase, implying that these neurons are likely inhibitory
975 motor neurons (35). Complementary analyses employed *Wnt1*-Cre to knock down *Glp1r* in the
976 mouse central and enteric nervous system (126). *Glp1r* mRNA transcripts were more abundant
977 in the muscle layer (including the myenteric plexus) than in the submucosal layer (including the
978 submucosal plexus). *Wnt1*-Cre enabled knockdown of more than 95% of *Glp1r* mRNA in the
979 ileal muscle layer, but only 80% in the jejunal and as low as 60% in the duodenal muscle layer
980 (126). Human myenteric plexuses were reported to contain GLP-1R immunoreactivity however
981 these experiments utilized an antibody, Ab39072 subsequently shown to lack sensitivity and

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

986

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

998

999 **Potential GLP1R expression in intestinal smooth muscle**

1000

1001 Whether GLP-1R agonism inhibits gut motility indirectly through neural mechanisms, or in part
1002 through direct interaction with a smooth muscle GLP-1R remains unclear (185-187). GLP-1
1003 may also reduce smooth muscle cell inflammation in the context of lipopolysaccharide-induced
1004 *Tnf* and *Il1a* expression in colonic smooth muscle cells isolated from BALB/c mice (188).
1005 Notably, ascertainment of definitive non-vascular smooth muscle cell GLP-1R expression

1006 remains challenging to date, as contamination from rare cell types within *ex vivo* preparations,
1007 including enteric neurons, cannot be excluded.

1008
1009 Thus, the local gut GLP-1 system is comprised of three main components: L cells as the main
1010 GLP-1 producer, and enteric neurons and IELs as the primary responders to GLP-1. Although
1011 GLP-1 secretion by L cells contributes to a majority of active GLP-1 levels in the circulation, it
1012 is worth mentioning that the local gut environment is frequently exposed to very high levels of
1013 GLP-1 (likely higher than the circulatory levels), especially when L cells are stimulated after
1014 meal ingestion or under various stress conditions. One can hypothesize that the presence of
1015 GLP-1R-expressing enteric neurons and IELs may present a feedback mechanism to modulate
1016 GLP-1 levels, as recently suggested (175). Moreover, local GLP-1 bioactivity is further modified
1017 by vascular and potentially enterocyte DPP-4 activity (189, 190). As enteric neurons and IEL
1018 subpopulations are difficult to isolate and study, the actions of GLP-1 on these cell types
1019 remains incompletely understood.

1020
1021

1022 **GLP-1 action in the immune system**

1023 GLP-1R agonists reduce systemic inflammation in preclinical and clinical studies, however the
1024 mechanisms linking GLP-1R signaling to modulation of immune cell activity remain uncertain
1025 (191). Low levels of *Glp1r* transcripts have been detected in isolated thymocytes, splenocytes,
1026 and lymph node cells from C57BL/6J mice by RT-PCR (192).
1027 *Glp1r* expression was also detected by RT-PCR in murine T cells, both in the basal state and
1028 after stimulation with α CD3/CD28, as well as in TH1 and TH17 polarized cells by RT-PCR,
1029 however levels were not compared relative to *Glp1r* expression in other cell types or tissues

1030 (193). Whole body *Glp1r*^{-/-} mice display normal cellularity and immunophenotypes in the thymus,
1031 spleen, lymph nodes and bone marrow, suggesting that expression of *Glp1r* is dispensable for
1032 the normal development of these immune organs (192). Recent studies have begun to explore
1033 the biology of GLP-1R expression in tissue-resident immune cells.

1034
1035

1036 **Invariant natural killer T cells as targets of GLP-1 action**

1037
1038 Invariant natural killer T (iNKT) cells exhibit properties of T cells and NKT cells; iNKT cells
1039 recognize the antigen-presenting CD1d that binds a variety of foreign lipids (194). A case report
1040 examined the putative direct effects of GLP-1 on human iNKT cells in the context of psoriasis.
1041 Two individuals with T2D and psoriasis treated with liraglutide exhibited reduced number of
1042 circulating iNKT cells after six weeks of treatment (195). Flow cytometric analyses revealed that
1043 55% of CD3⁺ T cells and 96% of TCR-Vα24⁺ iNKT cells showed positive intracellular staining
1044 using an unspecified PE-GLP-1R antibody, accompanied by increased levels of cAMP levels
1045 in these cells following incubation with GLP-1. Exogenous liraglutide treatment of high fat diet
1046 (HFD)-fed mice for five days reduced the number of iNKT cells in the blood and adipose tissues
1047 (196). Moreover, HFD-fed *Cd1d*^{-/-} mice, which lack iNKT cells in the body, lost less body weight
1048 when treated with liraglutide. Nevertheless, the expression of the GLP-1R in mouse iNKT cells
1049 was not definitively established and there is little rigorous evidence that B cells, NK, NKT, or
1050 innate lymphoid cells express the canonical GLP-1R.

1051
1052

1052 **GLP-1R expression in myeloid lineages**

1053

1054 Immunoreactive GLP-1R protein has been detected in mouse peritoneal macrophages by
1055 Western blotting using incompletely characterized antisera (197, 198), and in macrophage-like
1056 cell lines. However, full-length *Glp1r* transcripts have not been consistently detected in
1057 macrophages from mouse tissues or peritoneum, in the basal state or after induction of
1058 inflammation (20). Flow cytometry was used to investigate GLP-1R expression in peripheral
1059 blood from individuals with allergic asthma (199). About 10% and 5% of blood neutrophils and
1060 eosinophils, respectively, from healthy control subjects stained positive with an Alexa Fluor 700-
1061 conjugated GLP-1R antibody. The proportion of GLP-1R-immuno-positive eosinophils was
1062 reduced to 2% in subjects with asthma. The specificity of the GLP-1R antibody used in this
1063 study, AF700-GLP-1R, remains uncertain. Overall, the expression of GLP-1R has not yet been
1064 robustly confirmed in myeloid cells.

1065

1066 **GLP-1R transcripts in miscellaneous tissues**

1067

1068 ***Adipose tissue***

1069 *GLP1R* transcripts are reported in adipocyte cell lines ex vivo and in human visceral and
1070 subcutaneous adipose tissues by Taqman-based qPCR (200). Nevertheless, the precise GLP-
1071 1R+ cell type(s) within rodent or human adipose tissue, ranging from vascular ECs or VSMs,
1072 immune cells, or adipocytes themselves, has not been defined with certainty.

1073

1074 Iacobellis *et al.* detected *GLP1R* and *GLP2R* RNA by RNA-seq and qPCR in 8 human epicardial
1075 adipose tissue (EAT) samples from individuals with coronary artery disease with and without
1076 T2D (201). *GLP2R* mRNA transcripts were ~5-fold more abundant than *GLP1R* mRNA, *GLP1R*
1077 expression was described as “relatively low” however precise quantification or comparison with

1078 *GLP1R* expression in other tissues was not provided. GLP-1R-immunopositivity was detected
1079 in EAT and subcutaneous adipose tissue (SAT) from a diabetic and non-diabetic subject,
1080 analyzed by fluorescent IHC (Mab 3F52). Remarkably, in contrast to relatively comparable
1081 levels of *GLP1R* mRNA in EAT vs. SAT, GLP-1R immunopositive cells were much more
1082 abundant in EAT, compared to SAT (201). Tyramide signal amplification (which also has the
1083 potential to amplify signal from nonspecific primary antibody binding) was used to detect GLP-
1084 1R immunopositivity, due to the very low levels of GLP-1R expression, The precise identity of
1085 the GLP-1R+ cells in EAT was not determined. A follow-up study from the same group reported
1086 *GLP1R* expression in EAT by quantitative microarray analysis from 17 subjects undergoing
1087 coronary artery bypass grafting. Remarkably *GLP2R* mRNA transcripts were reported to be
1088 ~57-fold more abundant in EAT, relative to levels quantified for *GLP1R* (202).

1089

1090 ***Thyroid***

1091

1092 GLP-1R agonists produce thyroid C-cell hyperplasia in rats and mice (203, 204), consistent
1093 with expression of the GLP-1R in calcitonin-producing C cells within the rat and mouse thyroid
1094 (203). The development of C cell proliferation and calcitonin secretion in response to GLP-1R
1095 agonism in mice and rats is mediated by the canonical GLP-1R (204, 205) prompting
1096 considerable regulatory concern, and more detailed investigation of GLP-1R expression in the
1097 human thyroid. Out of 59 normal, hyperplastic and neoplastic human thyroid glands analyzed
1098 by IHC with the Mab 3F52 antibody, no GLP-1R-immunopositive follicular or C cells were
1099 detected in normal or hyperplastic thyroid tissue. Two of ten cases with sporadic medullary
1100 thyroid carcinoma (MTC) exhibited scattered GLP-1R-immunopositive C cells (51). Receptor
1101 autoradiography using ¹²⁵I-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,

1126 relative to expression in pancreas, kidney, gut, lung, and heart (10, 211). Indeed, Boland and
1127 colleagues did not detect *GLP1R* mRNA transcripts in human liver, Kupffer or stellate cell
1128 fractions, prepared from 3 human liver biopsy samples analyzed by bulk RNA-seq (212).
1129 Although a substantial number of studies report hepatocyte GLP-1R expression in mouse and
1130 human liver by immunocytochemistry or Western blotting, the great majority of these analyses
1131 use antisera that have not been validated for sensitive and specific detection of the GLP-1R
1132 (191). Moreover, isolated murine hepatocytes do not exhibit canonical cAMP responses to
1133 GLP-1R agonists, fail to bind fluorescent GLP-1 in vitro, and do not express the *Glp1r* (19-21,
1134 213).

1135

1136 *GLP1R* mRNA transcripts have been detected by RT-PCR in RNA isolated from human liver,
1137 and levels were reported to be reduced in livers from individuals with NASH (214). However,
1138 assignment of GLP-1R to hepatocytes was carried out using an antibody with unknown
1139 sensitivity and specificity. It seems likely that low level GLP-1R expression in liver reflects the
1140 presence of the GLP-1R in non-hepatocyte cells, such as immune cells or blood vessels.
1141 Indeed a fraction of ECs from mouse liver contained *Glp1r* mRNA transcripts detected by
1142 scRNA-seq (**Figure 5**) (155). Hence, it seems reasonable to conclude that the actions of GLP-
1143 1R agonists on hepatocytes are likely indirect, through incompletely identified pathways.

1144 **Concluding remarks for GLP-1 action outside the pancreas and nervous system**

1145 The availability data highlights multiple actions of GLP-1 and GLP-1R agonists, deduced from
1146 both preclinical and human studies, on key target cell types and tissues that do not exhibit
1147 robust GLP-1R expression. For example, GLP-1R agonists reduce the rates of myocardial
1148 infarction, decrease hepatic lipid accumulation, and attenuate renal albumin excretion, yet the
1149 GLP-1R is not expressed within most cardiomyocytes, hepatocytes, or renal glomerular and

1150 tubular epithelium. Similarly, GLP-1R agonism leads to reduced atherosclerosis, attenuated
1151 chylomicron secretion and decreased inflammation, yet the majority of VSMs, ECs, enterocytes
1152 and immune cells do not express the canonical GLP-1R. Collectively, these findings highlight
1153 the importance of indirect mediators of GLP-1 action, ranging from improvement of metabolic
1154 control, weight loss, and acute neural or hormonal effectors, as key contributors to actions
1155 ensuing from activation of the canonical GLP-1R.

1156
1157

1158 **Caveats and limitations**

1159

1160 Although considerable progress has been made in detecting GLP-1R expression, including
1161 characterization of two monoclonal antibodies suitable for detection of the primate and
1162 mouse/rat GLP-1R (3F52 and 7F38, respectively), it is still possible to incorrectly assign GLP-
1163 1R expression using these two antibodies. Importantly, the presence or absence of GLP-1R-
1164 immunopositivity remains highly technique- and tissue-dependent. Ideally, GLP-1R antisera
1165 should be regularly assessed for specificity under the conditions deployed for histochemistry or
1166 Western blotting using tissues from *Glp1r*^{-/-} mice, or with human cells or organoids engineered
1167 to delete the GLP-1R. Although a predominantly membrane pattern of cellular staining is ideally
1168 observed when using chemical probes or validated antisera to detect the GLP-1R (18, 44, 49),
1169 several reports of diffuse cytoplasmic staining raise questions about whether the antisera were
1170 used under conditions optimized for specificity. For example, the 7F38 antisera detected diffuse
1171 cytoplasmic and some membrane GLP-1R-immunopositivity throughout the entire islet (158),
1172 a pattern of staining which can be more difficult to interpret, relative to the more restrictive
1173 cellular localization of islet and membrane-localized GLP-1R expression. Indeed, in the context

1174 of establishing optimal technical conditions for the sensitive and specific detection of the human
1175 GLP-1R in human heart using IHC and the 3F52 antibody (18), we detected extensive
1176 cytoplasmic staining in tissues or cell types not known to express the GLP-1R. Hence, wherever
1177 possible, multiple complementary techniques should be used to interrogate the expression of
1178 the GLP-1R in specific cell types and tissues (18, 44, 49, 116, 118) including the use of negative
1179 controls and GLP-1R knockout tissues where applicable.

1180

1181 Many of the studies described herein utilize mice and rats as models for GLP-1 action and GLP-
1182 1R activation. The experimental conclusions may reflect the age, sex, species, housing
1183 conditions, diet, and health of the animals, as well as the specific experimental and technical
1184 approaches utilized. For example, the use of chemogenetic and optogenetic studies to
1185 interrogate PPG neurons informs the potential of broadly activating these neurons, but may not
1186 necessarily reflect the selective actions of GLP-1, acting through the GLP-1R, in the nervous
1187 system. Moreover, careful controls are required when using both these techniques, since
1188 optogenetic channels alter neuronal properties (215), and DREADD ligands such as clozapine-
1189 N-oxide can be converted to functional metabolites in the brain (216). Similarly, activation or
1190 deletion of the *Gcg* gene perturbs the expression of not only GLP-1, but multiple structurally
1191 related PGDPS, including oxyntomodulin and glucagon which may also signal through the GLP-
1192 1R. Hence, attribution of the results of these studies to the GLP-1/GLP-1R system should be
1193 done with caution. Furthermore, most studies localizing GLP-1 or the GLP-1R utilize post-
1194 mortem tissue and do not reveal information on GLP-1/GLP-1R expression in the live animal,
1195 or in live tissue.

1196

1197 This review did not examine the potential actions of GLP-1 degradation products, generated by
1198 enzymatic cleavage of GLP-1, which may produce multiple biological actions independent of
1199 the known GLP-1R (217). This biology may be relevant to circumstances characterized by
1200 pharmacological infusion of GLP-1, bariatric surgery, or GLP-1-producing tumors, and has been
1201 discussed elsewhere (6, 191). Moreover, although human islet GLP-1 action and GLP-1R
1202 expression has been extensively studied in the pancreas, much less is known about GLP-1R
1203 localization in other human organs and their cell types (Figure 8). As noted herein, the very low
1204 expression of the GLP-1R may preclude its reliable detection by RNA-seq, and more
1205 particularly in sc-RNA-seq, even in islet cells known to express the canonical GLP-1R and often
1206 used as a positive control (36). Hence, the old adage “absence of evidence is not evidence for
1207 absence” remains relevant for interpretation of these single cell or bulk data sets in tissues
1208 known to express low levels of the full length *GLP1R* mRNA. Along these lines, sensitive in situ
1209 transcript visualization techniques such as RNAScope and single molecular fluorescence ISH
1210 might prove transformative for understating GLP-1R mRNA localization and abundance in
1211 complex tissues.

1212
1213 Recent technological advances coupled with reagent development have fostered a more
1214 accurate and complete understanding of how and where endogenous and pharmacologically
1215 administered GLP-1 and GLP-1RA acts to control metabolism. Rapid ongoing developments in
1216 this field are likely to further refine existing insights, answering important mechanistic questions,
1217 while raising new testable hypotheses designed to illuminate the expanding direct vs. indirect
1218 cellular targets of GLP-1 action.

1219

1220	Abbreviations
1221	α SMA= α -smooth muscle actin
1222	AP=Area Postrema
1223	ARC=Arcuate nucleus
1224	bp=base pair
1225	CCK=Cholecystokinin
1226	cDNA=Complementary deoxyribonucleic acid
1227	cAMP=cyclic adenosine 3',5'-monophosphate
1228	CNS=Central Nervous System
1229	DPP-4=Dipeptidyl Peptidase-4
1230	DTA=Diphtheria Toxin
1231	DREADD= Designer Receptors Activated by Designer Drugs
1232	EAT=Epicardial Adipose Tissue
1233	EC=Endothelial cells
1234	EC ₅₀ =Effective concentration half maximal
1235	ENS=Enteric Nervous System
1236	EPAC=Exchange protein directly activated by cAMP
1237	ER=Endoplasmic Reticulum
1238	Ex4=Exendin-4
1239	Ex9=Exendin(9-39)
1240	FACS=Fluorescence-Activated Cell Sorting
1241	GABA= γ -aminobutyric acid
1242	GAD-Glutamic Acid Decarboxylase
1243	GCG=Glucagon gene or protein

1244	GFP=Green Fluorescent Protein
1245	GLP-1=Glucagon-like peptide-1
1246	GLP-1R=Glucagon-like peptide-1 receptor
1247	GLP-1RA= Glucagon-like peptide-1 receptor agonists
1248	HFD=High Fat Diet
1249	ICV=Intracerebroventricular
1250	IEL=Intestinal intraepithelial lymphocyte
1251	IHC=Immunohistochemistry
1252	iNKT cells=Invariant Natural Killer T cells
1253	ISH=In situ hybridization
1254	LA=Left Atrium
1255	LV=Left Ventricle
1256	Mab=Monoclonal Antibody
1257	Median Eminence
1258	MHC=Myosin Heavy Chain
1259	MTC=Medullary Thyroid Cancer
1260	NG2=Neural/glial Antigen 2
1261	NG=Nodose Ganglion
1262	NTS=Nucleus Tractus Solitarius
1263	OVLT=Organum vasculosum of the lamina terminalis
1264	PGDPs=Proglucagon-derived peptides
1265	PKC=Protein kinase C
1266	PLC=Phospholipase C
1267	PPG=Preproglucagon

1268 qPCR=quantitative PCR

1269 RA=Right Atrium

1270 RV=Right Ventricle

1271 RNA-seq=Ribonucleic acid sequencing

1272 scRNA-seq= Single cell Ribonucleic acid sequencing

1273 RT-PCR=Reverse Transcription-Polymerase Chain Reaction

1274 SiR=Silicon rhodamine

1275 SFO=Subfornical organ

1276 SSTR=Somatostatin Receptor

1277 SSTR2=Somatostatin Receptor 2

1278 SDA=Subdiaphragmatic vagal afferent deafferentation

1279 SAT=Subcutaneous Adipose Tissue

1280 TCR=T Cell Receptor

1281 TMR=Tetramethylrhodamine

1282 T2D=Type 2 Diabetes

1283 vGAT=vesicular GABA Transporter

1284 vGLUT2= Vesicular glutamate transporter 2

1285 VSM=Vascular smooth muscle cells

1286 YFP=Yellow Fluorescent Protein

1287

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1998 Figure Legends

1999

2000

2001 Figure 1: GLP-1 source, GLP-1R labeling and GLP-1R signalling in pancreatic islets

2002 A) Potential sources of GLP1 in the islets, as well as paracrine signaling pathways.

2003 Proglucagon products from α -cells that activate islet GLP-1Rs include glucagon and
2004 potentially GLP-1. The biological relevance of GLP-1 produced by α -cells remains an
2005 unresolved line of investigation.

2006 B) GLP1R labelling is observed at the membrane in β -cells. Predominantly cytoplasmic
2007 labelling is non-specific unless the GLP1R is agonist-bound in which case some surface
2008 and punctate intracellular staining is detected due to GLP1R internalization.

2009 C) Major GLP1R signaling pathways reported in primary rodent and human beta cells or
2010 islets. It is well established that GLP-1R signals through cAMP pathways, including PKA
2011 and EPAC2. β -arrestin 1 has been proposed to regulate GLP-1R signaling, however, the
2012 mechanisms of this pathway remain unresolved.

2013 D) Actions mediated by the GLP-1R in pancreatic islets.

2014 ADCY, adenylate cyclase, GLP1R, Glucagon-like peptide-1 receptor; PKC, protein kinase A;

2015 EPAC2, exchange protein directly activated by cAMP 2; INS, insulin SST, somatostatin.

2016

2017 Figure 2

2018 The UMAP (Uniform Manifold Approximation and Projection) plots were created to visualize
2019 gene expression in each cell. The x-axis and y-axis indicate the overall transcriptional difference
2020 of each cells arbitrated as "distance" in a two dimensional space; the closer the two cells on the
2021 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 marker 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 autonomic 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:

2046 periaqueductal grey; PBN: parabrachial nucleus; Pir: piriform cortex; PVN: paraventricular
2047 nucleus; PVT: paraventricular thalamus; Sep: lateral septum; SFO: subfornical organ; RPa:
2048 raphe pallidus; VLM: ventrolateral medulla; VMPO: ventromedial posterior nucleus.

2049 Figure 4

2050 GLP-1 receptor expression in heart and blood vessels within select organs and actions of
2051 GLP-1 associated with these organ and cell types. The arrow depicts the relative levels of
2052 GLP-1R expression deduced from human gene expression databases such as the Genotype
2053 Tissue Expression portal

2054 Figure 5

2055 Expression of mouse *Glp1r* in 40,449 murine endothelial cells. Count matrices were accessed
2056 on 19th Sep 2020 and reported by Kalucka et al. (219) (ArrayExpress ID E-MTAB-8077).

2057 Figure 6

2058 Co-expression of the proglucagon gene (*Gcg*) and enteroendocrine genes in 411 murine
2059 small intestine L cells. Count matrices were accessed on 19th Sep 2020 and retrieved from
2060 the scRNA-seq datasets reported by Gehart *et al* (167) (GEO accession number
2061 GSE113561). Mouse gene nomenclature: *Pyy*, Peptide YY; *Gip*, glucose-dependent
2062 insulintropic polypeptide; *Nts*, Neurotensin; *Tph1*, Tryptophan hydroxylase 1; *Sst*,
2063 Somatostatin; *Ghrl*, Ghrelin; *Sct*, Secretin; *Cck*, Cholecystokinin.

2064 Figure 7

2065 Expression of *Glp1r* in 4,974 murine ileal and colonic enteric neurons. Enteric neuron subtypes
2066 types were classified based on neuropeptide genes *Adcyap1* (PACAP neurons and *Gal*
2067 (galanin neurons). Count matrices were accessed on 19th Sep 2020 and aggregated from the
2068 scRNA-seq datasets reported by Drokhyansky et al (183) (Broad Institute Single Cell Portal

2069 SCP1038). Mouse gene nomenclature: *Adcyap1*, pituitary adenylate cyclase activating
2070 polypeptide or PACAP; *Gal*, galanin.

2071 Figure 8

2072 Schematic depiction of major targets of GLP-1 action, and the cell types within key organs
2073 that express the GLP-1 Receptor (GLP1R). The GLP-1 peptide is produced in and shown
2074 adjacent to the intestine, pancreas, and brain, the three major sites of GCG expression.

2075 EC=Endothelial Cells; IEL=Intestinal Intraepithelial Lymphocytes; SA node=Sinoatrial node;
2076 VSMC=Vascular Smooth Muscle Cell.

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Cell type	<i>Glp1r/GLP1R</i>	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

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

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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)

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