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Review

MOLECULAR
METABOLISM

Glucagon-like peptide 1 (GLP-1)

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ABSTRACT

Background: The glucagon-like peptide-1 (GLP-1) is a multifaceted hormone with broad pharmacological potential. Among the numerous metabolic effects of GLP-1 are the glucose-dependent stimulation of insulin secretion, decrease of gastric emptying, inhibition of food intake, increase of natriuresis and diuresis, and modulation of rodent β-cell proliferation. GLP-1 also has cardio- and neuroprotective effects, decreases inflammation and apoptosis, and has implications for learning and memory, reward behavior, and palatability. Biochemically modified for enhanced potency and sustained action, GLP-1 receptor agonists are successfully in clinical use for the treatment of type-2 diabetes, and several GLP-1-based pharmacotherapies are in clinical evaluation for the treatment of obesity.

Scope of review: In this review, we provide a detailed overview on the multifaceted nature of GLP-1 and its pharmacology and discuss its therapeutic implications on various diseases.

Major conclusions: Since its discovery, GLP-1 has emerged as a pleiotropic hormone with a myriad of metabolic functions that go well beyond its classical identification as an incretin hormone. The numerous beneficial effects of GLP-1 render this hormone an interesting candidate for the development of pharmacotherapies to treat obesity, diabetes, and neurodegenerative disorders

1. FROM THE DISCOVERY OF INSULIN TO THE DISCOVERY OF GLP-1

Maintenance of adequate glucose metabolism is a prerequisite for human health, and pathological failure to buffer against prolonged episodes of hypo- and/or hyperglycemia can result in severe microvascular disease, metabolic damage, coma, and death. Unsurprisingly, before the discovery and commercialization of insulin in the 1920's, juvenile-onset diabetes, with its paucity of endogenous insulin, was a disease with only a few years between a patient's diagnosis and premature demise. The discovery of insulin and its ability to lower blood glucose transformed juvenile-onset (type-1) diabetes from a fatal to a manageable disease. However, early on, it was noted that insulin derived from pancreatic extracts [1] or as crude insulin preparations [2] sometimes first elevated blood glucose and then later decreased blood glucose levels. The increase in blood glucose, which peaked around 20 min after the administration, was believed to be caused by a toxic fraction resulting from suboptimal insulin purification [2]. The same toxic fraction was thought to be responsible for local skin irritations and abscesses that were sometimes observed in patients treated with these formulations [2]. These observations spurred efforts to optimize the isolation and purification of insulin from tissue homogenates.

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Review

Aiming to develop a fast and inexpensive method for commercial insulin purification, in 1923, Charles Kimball and John Murlin precipitated a pancreatic fraction that, after evaporation and reconstitution in water, had a robust hyperglycemic effect when injected into rabbits and dogs [3]. Because the fraction was incapable of decreasing blood glucose, Kimball and Murlin hypothesized that the hyperglycemic effect resulted from a secreted factor, one that antagonizes insulin's hypoglycemic effect. The factor was named '*the glucose agonist*', or "*glucagon*" [3]. Over the subsequent decades, substantial research efforts were directed toward unravelling the molecular underpinnings of glucose regulation by the two opposing pancreatic hormones (as reviewed in [4,5]). Among the numerous major discoveries made in this regard was the finding that the hyperglycemic effect of glucagon resides in its ability to act in the liver to stimulate glycogenolysis and gluconeogenesis [6–12] despite its seemingly paradoxical ability to stimulate insulin secretion in humans reported by Ellis Samols in 1965 [13]. In the early 1950's, Earl Sutherland and Christian de Duve demonstrated that pancreatic glucagon production was abolished when the function of islet α-cells was compromised by treatment with either cobalt or synthalin A but was preserved upon alloxan-induced impairment of the exocrine pancreatic acinar and islet β-cells [14,15]. Subsequent histological studies by Claes Hellerstrom and Bo Hellman divided α-cells into α_1-and α_2-subtypes, but it was not until the early 1960s that convincing evidence was provided that α_2-cells were the source of glucagon [16]. This conclusion was based on suppression of α_2-cell numbers in rats and guinea pigs by administration of exogenous glucagon [17,18] plus the strong staining in α_2-cells for tryptophan, which is an amino acid present in glucagon. Later, α_1-cells were shown by Bo Hellman and Ake Lernmark to be the source of an inhibitor of insulin secretion [19]. This was eventually established as somatostatin.

In 1959, Roger Unger generated and characterized the first glucagon-detecting antibody and thus paved the path to the development of the first radioimmunoassays (RIA) to detect glucagon in blood and tissue samples [20]. It was subsequently found by Ellis Samols and Vincent Marks in 1966 [21] and confirmed by others that glucagon-like immunoreactivity was also present in extra-pancreatic tissues, in particular in the intestine [22–27]. Notably, the circulating glucagon-like material was also detected in 1967 by Samols and Marks in pancreatectomized humans [21] and in the following year by Roger Unger and Isabel Valverde in dogs [27]. This eliminated the pancreas as the origin of this glucagon-like immunoreactivity. Unger then demonstrated in 1968 that intraduodenal, but not intravenous, glucose administration increases circulating glucagon-like immunoreactivity [27], implying that the intestine secretes this glucagon-like material. The intestinal glucagon-like material was heterogeneous, comprising several fractions of different molecular size and with apparently distinct biological actions relative to pancreatic-derived glucagon [27,28]. The intestinal glucagon-immunoreactive material did not induce hyperglycemia when injected into dogs and was devoid of glycogenolytic effects in the isolated perfused rat liver [27]. However, analogous to the effects of glucagon, the intestinal material stimulated the release of insulin, suggesting that the intestinal glucagon-like fractions are either different substances or different forms of pancreatic glucagon [27]. Further corroborating the distinct nature of the intestinal and pancreatic glucagon-like material, immunocytochemical studies revealed that the intestinal cells that stained positive for the glucagon antibody differed from the pancreatic glucagon-producing α-cells in terms of their morphology and ultrastructure [29], classifying these intestinal cells as "L-cells" [30]. Glucagon-like immunoreactive material of larger size than of glucagon

was subsequently detected in the pancreas of dogs [31] as well as in islets isolated from birds [32] and guinea pigs [33]. Collectively, these studies suggested that glucagon might originate from a larger precursor molecule that is post-translationally cleaved into several fractions of distinct size and with different functions. Immunoprecipitation analysis of rat pancreatic islets identified this precursor as an 18,000 molecular weight (MW) protein, now classified as proglucagon [34]. In the pancreas, proglucagon was found to be cleaved to produce two fragments, mature glucagon and a 10,000-MW second protein. This latter protein lacked the glucagon sequence and was named major proglucagon fragment (MPGF) [34,35] (Figure 1).

In the early 1980s, it was established that the major form of the intestinal glucagon-immunoreactive material, a peptide designated glicentin, contained the full glucagon sequence [36–40], and glicentin was proposed to represent at least a fragment of proglucagon, because it was also identified in the pancreas [41]. Although glicentin was initially thought to comprise ∼100 amino acids, its purification from porcine intestine established glicentin as a 69-amino acid peptide [39]. Thus, intestinal proglucagon is cleaved into distinct fractions that are different from those derived in the pancreas. In 1982, a smaller intestinal form was identified as a 37-amino acid peptide containing the full 29-amino acid sequence of glucagon with 8 additional amino acids on its C-terminal end [36], the same as found in the C-terminus of glicentin. Based on its potency to act on oxyntic glands, the 37-amino acid peptide was named oxyntomodulin [42] (Figure 1). Thus, it appeared that proglucagon undergoes a tissue-specific, differential processing leading to the formation of glicentin and oxyntomodulin in the gut and to glucagon plus the N-terminal fragment of glicentin in the pancreas [34,43] (Figure 1). By using a method that allows the prediction of a protein sequence through decoding of recombinant cDNA clones, at the beginning of the 1980s Joel Habener established that a different glucagon-related peptide is encoded within the anglerfish preproglucagon cDNA [44–46]. Two glucagon-related peptides were subsequently identified in the rat [47,48], hamster [49], bovine [50], and human [51] proglucagon sequence. The two hypothetical peptides were designated glucagon-like peptides 1 and 2 (GLP-1 and GLP-2) [49] (Figure 1). Radioimmunoassays in tissues from rats [52], pigs [53], and humans [54] then established a distinct profile of proglucagon-derived peptides (PGDPs) in the pancreas and the intestine with production of glucagon and one large fragment (major proglucagon fragment; MPGF) in the pancreas and liberation of smaller GLP-1 immunoreactive peptides in the intestine.

2. IDENTIFICATION OF GIP AND GLP-1 AS GASTROINTESTINAL INSULINOTROPIC HORMONES

In 1902, Ernest Bayliss and William Starling identified a substance that is produced in and secreted from the epithelial cells of the duodenum in response to the contact of these cells with acidic chyme [55]. Bayliss and Starling noted that the substance, after being released into the circulation, stimulates the pancreas to secrete pancreatic juice; they named the substance secretin, thereby identifying the first gastrointestinal hormone [55]. In 1906, Benjamin Moore reported that the glucosuria of diabetic patients could be ameliorated by repeated oral administration of a pig-derived intestinal mucosal homogenate [55]. Influenced by the work of Bayliss and Starling, Moore hypothesized that the intestinal mucosa membrane produces a substance of the nature of a hormone that decreases blood glucose via its stimulatory action on the pancreas [55]. Supporting Moore's hypothesis, in 1929, Edgard Zunz and Jean LaBarre isolated a fraction from gut extracts that decreased blood glucose in experimental animals [56]. Assuming that
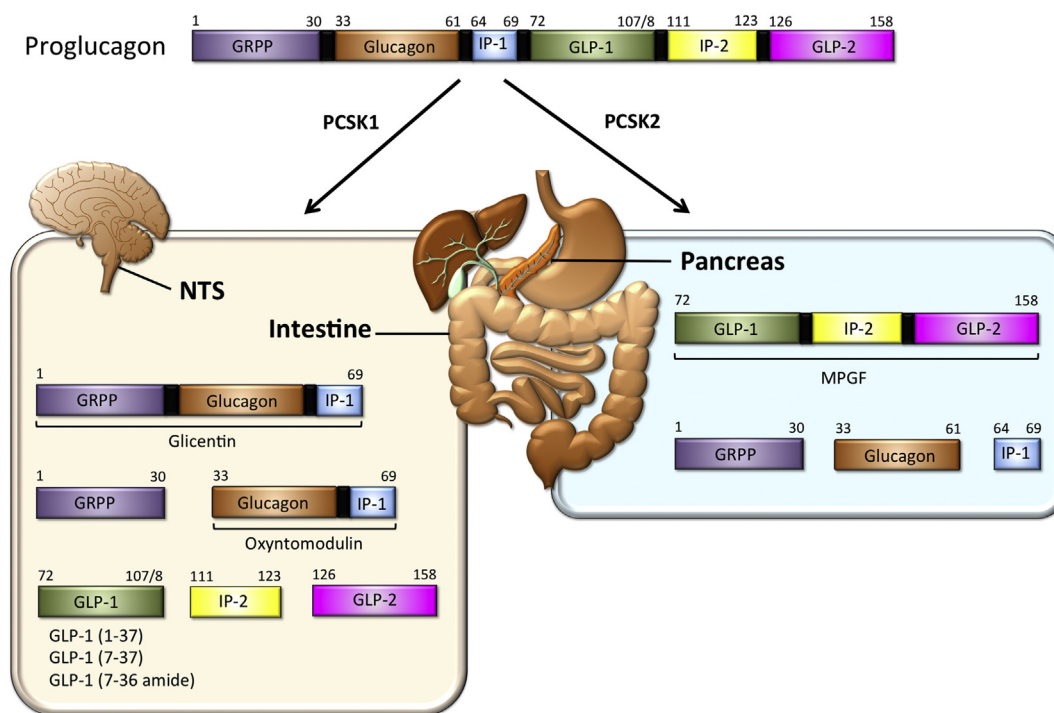
Figure 1: Schematic on the tissue-selective processing of proglucagon. PCSK1: prohormone convertase 1/3; PCSK2: prohormone convertase 1/3; NTS: nucleus tractus solitarii; GRPP: glicentin-related polypeptide; IP-1 intervening peptide-1; IP-2: intervening peptide-2, MPGF: major proglucagon fragment; GLP-1: glucagon-like peptide-1; GLP-2: glucagon-like peptide-2. See text for further explanations.

the hypoglycemic effect of this fraction resides in its ability to stimulate the endocrine pancreas to secrete insulin, the factor was named incretin [56,57].

In the following years, research directed towards identification of insulinotropic intestinal factor(s) was hampered by difficulties in quantifying insulin from tissue and blood samples. Once the first insulin radioimmunoassays (RIA) became available in the 1960's [58,59], the insulinotropic action of intestinal mucosa extracts was confirmed in healthy humans and was demonstrated to be absent in individuals with type-1 diabetes [60]. Several nearly simultaneous reports then demonstrated that glucose-stimulation of insulin secretion was much greater when glucose was given orally as compared to parenterally [61,62]. Greater insulinotropic action of oral glucose relative to its peripheral administration was demonstrated for both normal weight and obese individuals [63] and henceforth became known as the incretin effect [64,65]. In 1965, Ellis Samols and colleagues had already hypothesized that the intestinal glucagon-like material might somehow be related to the incretin effect [66].

Between 1969 and 1971, work by John Brown and Raymond Pederson, together with the peptide chemistry expertise of Erik Jorpes and Viktor Mutt [67], led to the identification of the gastric inhibitory polypeptide (GIP), a 42-amino acid polypeptide that was named based on its potency to suppress gastric motility and gastric acid secretion [68—70]. In 1973, John Dupré demonstrated in healthy human volunteers that GIP, when given intravenously at near-physiological doses together with glucose, potentiates immunoreactive insulin and improves glucose tolerance, thus identifying GIP as a contributor to the incretin effect [71]. The insulinotropic action of GIP was subsequently demonstrated to be due to direct action on the pancreas, where it enhances glucose-stimulation of insulin secretion, as demonstrated in isolated rat islets [72—75], and in the perfused pancreas of dogs [76] and humans [77].

Work from Werner Creutzfeldt's laboratory in 1983 demonstrated that removal of GIP from gut extracts by immunoprecipitation diminishes the incretin effect by less than 50% [78]. Studies in humans with resection of different parts of the small intestine demonstrated that the incretin effect and the GIP response to oral glucose did not correlate — the incretin effect was better correlated with preservation of the ileum [79]. Because glucagon was known to stimulate insulin secretion [13], it was natural to look at the newly identified glucagon-like peptides that possessed ~50% sequence homology to glucagon. Indeed, the glucagon-like peptide that was identified in the anglerfish, as well as the mammalian proglucagon cDNA and both resembled the GIP sequence, suggesting that these proglucagon products might have insulinotropic effects [44—46]. Neither GLP-1 (1-36amide) nor GLP-2, peptide fragments corresponding to the predicted structures from the gene sequence, were active on insulin secretion, but a truncated version of GLP-1 extractable from human and porcine gut was subsequently found to enhance insulin secretion in various experimental models [80—82] and, ultimately, in human studies, i.e. GLP-1 was identified as a potential incretin hormone [83].

3. TRANSCRIPTIONAL REGULATION OF PREPROGLUCAGON (GCG)

Preproglucagon (Gcg) is expressed in pancreatic α-cells, in enteroendocrine L-cells throughout the gut, predominantly in the distal ileum and colon, and in a population of neurons in the nucleus tractus solitarii (NTS) of the brainstem [52,84—89]. Diphtheria toxin-induced ablation of preproglucagon-positive neurons in the NTS

Review

demonstrated that this small population of neurons is the primary source of endogenous GLP-1 in the brain [90].

Cleavage sites within the proglucagon molecule together with expression of specific prohormone convertase enzymes determines which smaller peptide molecules/hormones are formed, including glicentin (aa 1—69), glicentin-related pancreatic polypeptide (GRPP; aa 1—30), glucagon (aa 33—61), oxyntomodulin (OXM; aa 33—69), the major proglucagon fragment (MPGF; aa 72—158), and the glucagon-like peptides 1 (GLP-1; aa 72—107/108) and 2 (GLP-2; aa 126—158) (Figure 1) [51—53,91]. Several of the PGDPs have important and well-defined (pharmacological) effects on systemic metabolism, including the modulation of food intake and satiety (GLP-1, glucagon, oxyntomodulin), regulation of fluid homeostasis (water intake and urine excretion) (GLP-1) [92], thermogenesis (glucagon), lipid metabolism (GLP-1, glucagon, GLP-2), gastrointestinal motility (glucagon, GLP-1, GLP-2), and gastric emptying (glucagon, GLP-1, GLP-2). Glucagon released from pancreatic α-cells and GLP-1 released from intestinal L-cells have opposing effects on blood glucose. Therefore, the expression and cleavage of proglucagon and secretion of the various PGDPs must be precisely controlled in a cell-specific process.

The expression of Gcg in the pancreas, intestine, and brain is under the control of a single promoter and is initiated from an identical transcription start codon (Figure 2). The rodent *Gcg* promoter, along with its adjacent DNA control/enhancer elements, is located within the 2.5 kb 5′-flanking region of the *Gcg* transcription start [89,93,94]. In rodents, the ∼1.3 kb 5′-flanking sequence is sufficient to direct transgene expression to Gcg + cells in the brain and the pancreas [95] but extension of this region to include ∼2.5 kb is required to target Gcg + cells in the intestine [96], including evolutionarily preserved sequences in the first intron [97].

The cell-specific expression of Gcg is orchestrated by a series of homeodomain proteins that bind to specific cis-acting elements in the Gcg promoter and/or enhancer region to either stimulate or inhibit Gcg promoter activity [94,98—100]. The rat *Gcg* promoter comprises at least 5 cis-acting elements ($G_1 - G_5$) plus a cAMP response element (CRE), all of which are located within the 2.5 kb region upstream of the *Gcg* transcription start [89,94,101—103]. In α-cells, the TATA box, as well as the adjacent G1 and G4 elements, represent the minimal promoter which is essential for *Gcg* expression while the elements G5, G2, G3, and CRE represent a more distal located enhancer region [94,101—103] (Figure 2).

Signaling events leading to the stimulation of *Gcg* expression in α-cells include heterodimerization of the transcription factor paired box protein 6 (Pax6) with cellular muscular aponeurotic fibrosarcoma (c-Maf), MAF bZIP transcription factor B (MafB) or caudal type homeobox 2/3 (Cdx2/3), and consequent binding of these heterodimers to the G1 element (Figure 2) [98,104—106]. Pax6 can also bind to the G3 element [100], and it plays a key role in regulating *Gcg* expression and α-cell
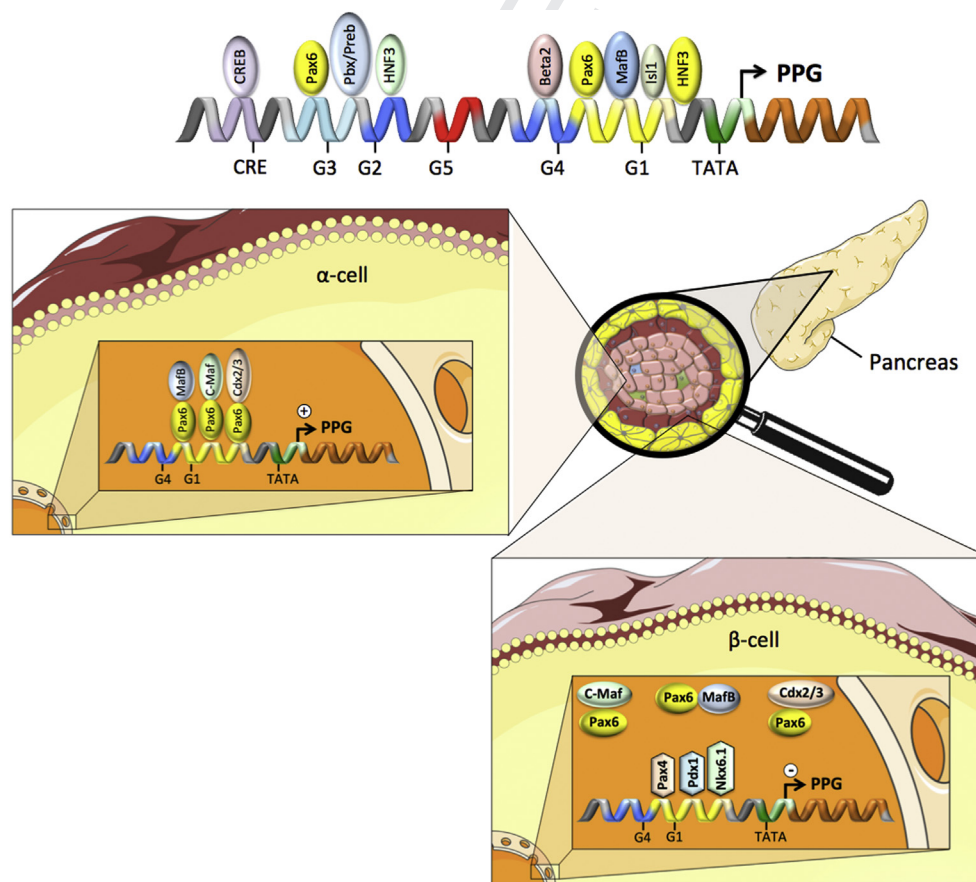

Figure 2: Schematic on the tissue-selective processing of proglucagon in the pancreatic islets. Schematic on the transcriptional regulation of preproglucagon (PPG) in the pancreatic islets. Pax6: paired box 6; CDX2/3: caudal type homeobox 2/3; MafB: MAF bZIP transcription factor B; cMaf: c-Maf inducing protein; NKX2.1: NK2 homeobox 1; PDX1: pancreatic and duodenal homeobox 1; Pax4: paired box 4; CRE: cAMP response element; CREB: cAMP response element binding protein; PPG: preproglucagon; HNF3: hepatocyte nuclear factor 3; Isl1: ISL LIM homeobox 1; Preb: prolactin element binding. For further explanations, please see text.

development, because mice lacking Pax6 fail to produce glucagon-producing α-cells [107]. Pax6 also stimulates Gcg expression in the enteroendocrine cells of the gastrointestinal epithelium [108]. Mice homozygous for a dominant negative Pax6 mutation (SEYNeu) have repressed Gcg expression in enteroendocrine cells in the small and large bowel and absence of immunoreactive GLP-1 and GLP-2 [109]. Further supporting the role of Pax6 in regulating intestinal Gcg expression, adenoviral overexpression of Pax6 enhances Gcg promoter activity and Gcg expression in intestinal enteroendocrine cells such as the secretin tumor cell line-1 (STC-1) and cells derived from colonic tumors of transgenic mice expressing large T antigen under the control of the *proglucagon* promoter (GLUTag cells) [108].

Other transcriptional mechanisms regulating Gcg expression in α-cells include interaction of Cdx2/3, POU domain transcription factor brain 4 (Brn-4), hepatocyte nuclear factor 3 alpha (HNF3α; a.k.a. Foxa1), hepatocyte nuclear factor 3 beta (HNF3β; a.k.a. Foxa2), paired box protein 2 (Pax2), neuronal differentiation factor 1/beta 2 (NeuroD/Beta2), and basic helix-loop-helix transcription factor E47 with the G1, G2, G3, or G4 elements (Figure 2) [100,102,104,106,110—118]. Emphasizing their role in regulating glycemia, mice lacking Foxa1 or Foxa2 die shortly after birth due to severe hypoglycemia and substantial reduction in Gcg mRNA levels in the pancreas [119,120]. Notably, α-cell development is impaired in mice lacking Foxa2 but is normal in mice lacking Foxa1 [119,120]. These data suggest that Foxa1 affects glucagon levels via its action on the Gcg promoter, while Foxa2, in addition to modulating Gcg promoter activity, affects α-cell differentiation. Interestingly, β-cell specific deletion of Foxa2 also results in postnatal death due to severe hypoglycemia, but the low glucose level in Foxa2-negative mice seems to be caused by hyperinsulinemia rather than by changes in Gcg expression [121]. The LIM homeobox protein 1 (Isl1) is ubiquitously expressed in mature endocrine cells of the pancreas [122], and its ablation in mice results in failure to develop any pancreatic endocrine cell type [123]. In Chinese Hamster insulinoma InR1-G9 cells, Isl1 enhances the activity of the Gcg promoter [118]. Isl1 also interacts with the enhancer region of the genes encoding for insulin [124] and somatostatin [125], stimulating their transcription. In islet and intestinal endocrine cell lines, Cdx2/3 (the same protein is characterized as Cdx2 in mice and Cdx3 in hamsters) activates Gcg gene transcription via binding to the G1 element of the Gcg promoter [98]. Overexpression of Cdx2 in α-cell InR1-G9 cells accordingly increases Gcg expression [113].

In both α-cells and intestinal L-cells, Gcg expression is controlled by certain homeodomain proteins [94,98,100] and by cAMP-activation of protein kinase A (PKA), as demonstrated in primary rat intestinal cultures [126], isolated pancreatic cell lines [127,128] and enteroendocrine GLUTag and STC-1 cell lines [129,130]. In STC-1 cells, disruption of the CRE element in the Gcg enhancer only partially blunts PKA-stimulation of Gcg expression [130,131]. This suggests that PKA also affects Gcg transcription via CRE-independent mechanisms. Consistent with this, certain effectors of the Wnt pathway, including lithium and β-catenin, enhance Gcg expression in intestinal but not pancreas-derived cell lines, supposedly via inhibition of the glycogen synthase kinase-3beta (GSK-3beta) [132,133]. PKA has been demonstrated to inhibit GSK-3beta [134,135], indicating that the CRE-independent PKA stimulation of the Gcg promoter might be mediated via negative regulation of the Wnt pathway [132,133]. Other factors stimulating Gcg expression in the intestine include protein hydrolysates [101] and insulin [133]. Insulin stimulation of intestinal Gcg promoter activity and of GLP-1 secretion [133] is noteworthy because insulin inhibits glucagon production and secretion in isolated rat islets [136] and in hamster islet InR1-G9 cells [137]. While the mechanism underlying insulin inhibition of pancreatic glucagon production warrants further clarification, it is thought to be achieved by through transcriptional mechanisms via an insulin-responsive element (IRE) in the Gcg promoter of the α-cells [138].

Notably, whereas Gcg is robustly expressed in α-cells, its expression is suppressed in β-cells through binding of pancreatic and duodenal homeobox 1 (Pdx1), Pax4, and homeobox protein Nkx6.1 to the G1 element, thereby competitively preventing the binding of Pax6/Maf heterodimers to the G1 element (Figure 2) [100,139,140]. While substantial evidence supports a role of Pdx1 in the negative regulation of Gcg expression, Pdx1 immunoreactivity has also been demonstrated in some Gcg expressing L-cells [141]. Overexpression of Pdx1 alone is insufficient to block Gcg expression in α-cell cultures (αTC-1 cells), isolated murine islets, or GLUTag enteroendocrine cells [142]. These data collectively suggest that Pdx1 requires interaction with other transcription factors to inhibit Gcg expression.

In summary, the cell-type selective expression of Gcg is regulated through more than a dozen transcription factors that selectively bind to cis-acting elements in the Gcg promoter and enhancer regions, thereby either stimulating or inhibiting Gcg expression. Apart from a series of homeodomain proteins, Gcg expression is also stimulated by PKA in response to increased levels of cAMP [94,99,101,129]. Insulin stimulates intestinal Gcg expression [133] while at the same time inhibiting Gcg expression in α-cells [89,137,138]. Certain effectors of the Wnt pathway further enhance Gcg expression in the intestine but not in the pancreas [94,132,143].

4. POSTTRANSLATIONAL PROCESSING OF PREPROGLUCAGON

The vast majority of glucagon is produced in the pancreatic α-cells, but, under some conditions, small amounts of glucagon also have been detected in the intestinal L-cells [87], although the validity of immunoassays to distinguish different proglucagon products has been questioned, and some reports were unable to find genuine glucagon in the intestine by mass spectrometric analysis [144—146]. Glucagon also has been detected immunohistochemically in certain Gcg-positive neurons of the NTS [84—86]. The tissue-specific cleavage of proglucagon is orchestrated by the selective expression of the prohormone convertase (PC) enzymes. Prohormone convertase 1 (PC1; a.k.a. PCSK1 or PC1/3) is expressed in GCG + cells in the brain and the intestine, and cleavage of Gcg by PCSK1 results in the liberation of GLP-1, GLP-2, glicentin, oxyntomodulin, and IP2 (Figure 1) [147—150]. In contrast, PC2 (a.k.a. PCSK2) is highly expressed in the pancreas [151], and its expression in α-cells results in cleavage of Gcg into "pancreatic type" glucagon, GRPP, MPGF, and a small intervening peptide (IP1). Studies in the porcine and human pancreas suggest that the PCSK2-liberated PGDPs are all co-secreted in equimolar concentrations from the islets [152,153]. Underlining the role of PCSK2 in liberation of glucagon via proglucagon processing, PCSK2-deficient mice are slightly hypoglycemic upon fasting, have a reduced rise in blood glucose following intraperitoneal glucose administration, display impaired processing of Gcg in the α-cells, and develop α-cell hyperplasia [154,155]. The hypoglycemia and α-cell hyperplasia seem to directly result from glucagon deficiency because continuous intraperitoneal glucagon supplementation is sufficient to correct the hypoglycemia and the α-cell hyperplasia of Pcsk2$^{-/-}$ mice [156].

While PCSK2 is the predominant prohormone convertase in α-cells in non-pathological conditions, α-cell PCSK1 immunoreactivity increases in rodent models of metabolic stress. α-cell PCSK1 activity and/or expression is found in embryonic and neonatal mice, with pregnancy, and in models of prediabetes and diabetes [157—160]. In cultured α-

Review

cell lines or isolated islets, high-media glucose concentrations increase PCSK1 expression and cellular GLP-1 content [161,162]. Islet GLP-1 production is also mediated by the cytokine IL-6, which is released in response to exercise, obesity and diabetes [163,164]. Lastly, upon streptozotocin-induced destruction of the β-cells, there is an acute increase in islet PCSK1 and Gcg expression and increased processing of proglucagon to GLP-1 [158]. Consistent with this, adenoviral overexpression of PCSK1 in α-cells increases islet GLP-1 production and secretion, ultimately leading to enhanced glucose-stimulation of insulin secretion and improved survival of the islets [165]. Further, glucagon receptor KO mice also have compensatory increases in α-cell GLP-1, and GLP-1 receptor (GLP-1R) signaling has been reported to contribute to the preserved glucose responses after streptozotocin administration [166,167]. Together, these data point to a potential role of the α-cells in compensating for increased β-cell functional demand under conditions of insulin resistance, pregnancy, and cellular stress through intra-islet GLP-1 production [168,169]. Multiple lines of evidence are accumulating that challenge current dogma and imply that pancreatic GLP-1 production also has a role, under some circumstances, in regulating insulin secretion via paracrine action [170—172]. Further work is needed to understand the pathological and physiological role for GLP-1 vs. glucagon and paracrine signaling in islet cell development. Glucagon-stimulation of insulin secretion is preserved in islets isolated from β-cell-specific GCGR KO mice but is attenuated upon treatment of these islets with exendin (9—39) [173]. Consistent with this, glucagon-stimulation of insulin secretion is decreased in islets isolated from β-cell-specific GLP-1R KO mice [173]. These data underscore the importance of α-to β-cell communication and indicate that glucagon may be the dominant PGDP acting via the GLP-1R on the β-cells to stimulate insulin secretion [173].

Several forms of GLP-1 are processed from proglucagon and vary in their ability to enhance glucose-induced insulin secretion. The different forms include GLP-1 (1—37) (or 1-36amide) and two "truncated" forms, GLP-1 (7-36amide) ("amidated GLP-1) and GLP-1 (7—37) ("glycine-extended GLP-1") (Figure 1) [174]. In humans, nearly all circulating GLP-1 is one of the truncated forms, with ∼80% of GLP-1 immunoreactivity corresponding to GLP-1 (7-36amide) and ∼20% to the glycine-extended GLP-1 (7—37) [175]. The relative abundance of GLP-1 (7-36amide), GLP-1 (7—37) and GLP-1 (1—37) differs among species [176—178]. Both the longer and the truncated forms of GLP-1 are detected in extracts of rat intestine and pancreas [52], in a rat Gcg-producing cell line [179] and upon transfection of rat pituitary or rat insulinoma cells with a glucagon fusion gene [180]. While GLP-1 (7-36amide) and GLP-1 (7—37) are equally potent to stimulate the secretion of insulin and c-peptide [181], GLP-1 (1—37) has a much lower insulinotropic efficacy [80—82].

5. GLP-1 DEGRADATION

Native GLP-1 has a very short half-life, which, depending on the species, is around 1—2 min [182—184] and results from two causes: (a) the action of the enzyme dipeptidylpeptidase-4 (DPP-4) and (b) renal elimination. DPP-4 cleaves GLP-1 (7-36amide) and GLP-1 (7—37) at the N-terminal dipeptide to generate GLP-1 (9-36amide) or GLP-1 (9—37), low affinity ligands for the GLP-1 receptor [185—188]. Both these intact forms as well as inactivated GLP-1 metabolites are also rapidly cleared from the circulation via the kidneys. In mice, the enzyme neprilysin additionally rapidly degrades the metabolites, making GLP-1 difficult to measure in this species [189]. While GLP-1 degradation is unaffected by kidney function, the clearance of both

GLP-1, and to a greater extent its inactive metabolites, is delayed in patients with renal insufficiency [184].

DPP-4 exists in two forms; i.e., it is a membrane-spanning cell surface protein and a circulating protein, and both forms have actions that extend beyond its proteolytic activity [190]. In the intestine, DPP-4 is highly expressed in the enterocyte brush border and in endothelial cells [191]. Consequently, as discussed in a comprehensive review [152], a large portion of intestinal GLP-1 is already degraded in the capillaries of the distal gut with an estimated ∼25% of active GLP-1 reaching the liver and only ∼10—15% reaching the general circulation [152,176,183,191]. Pharmacological inhibition or genetic reduction of DPP-4 activity preserves much higher circulating levels of intact GLP-1 [191,192], and this was demonstrated to potentiate the insulinotropic effect of GLP-1 in anesthetized pigs (eventually leading to the development of DPP-4 inhibitors for clinical use) [193]. When administered i.v., i.p., or s.c. in rats, GLP-1 (7—36 amide) has a half-life of 0.8—4.7 min, 0.6—13.5 min and 4.6—7.1 min, respectively [194].

Substantial evidence indicates that the DPP-4-generated GLP-1 metabolites (GLP-1 (9-36amide) and GLP-1 (9—37)) have no major role in regulating glucose metabolism [195—197]. However, one report of an experiment in obese humans suggested that GLP-1 (9-36amide) is a weak insulin secretagogue [198], and administration of GLP-1 (9-36amide) improves glucose handling without affecting insulin secretion in anesthetized pigs and in humans [199,200]. GLP-1 (9-36amide) also improves cardiac output in the post-ischemic mouse heart when administered during reperfusion, and it affects vasodilation in mesenteric arteries in mice [201]. This scenario contrasts with the apparent lack of effect of high doses of GLP-1 (9-36amide) on glucoregulation in ob/ob mice or on cognitive function in high-fat fed mice [202,203]. Indeed, there is evidence that truncated GLP-1 (9-36amide) has no effect on glucose clearance or insulin secretion in healthy humans [196]. In fact, the peptide acts as a weak GLP-1R antagonist, clearly counteracting the biological effects of GLP-1 (7-36amide) in vitro [204].

6. REGULATION OF GLP-1 SECRETION

6.1. Intestinal distribution of the L-cells

Although there is some species variation, the density of L-cells is relatively low in the proximal small bowel and increases distally along the gut, with greatest density in the ileum and colon [205—209]. In rats, pigs, and humans, the majority of L-cells are located in the epithelial layer of the distal bowel [208]. The apical surface of the L-cell faces the gut lumen, where it has direct contact with luminal nutrients (Figure 3) [208]. Consistent with this, plasma levels of GLP-1 rapidly increase in healthy humans upon direct administration of carbohydrates or lipids into the ileum [210]. While potentially being sufficient for the early rise in circulating GLP-1 upon food intake, the relative scarceness of L-cells in the proximal small intestine of both rodents and humans [208] suggests that proximal to-distal neuronal and/or humoral signals might affect the release of GLP-1 during meals [208], in particular during the early phase of GLP-1 secretion. Nonetheless, the ability of L-cells to secrete GLP-1 in direct response to luminal nutrients certainly plays an additional role, and may be the main mechanism of GLP-1 secretion. Contact of nutrients with L-cells in the distal gut likely explains the prolonged duration of elevated GLP-1 during meals, and it might also be responsible for the typically observed increased levels of GLP-1 following gastric bypass and, albeit less so, after sleeve gastrectomy surgery [211—216], because surgical repositioning of the distal gut more rapidly exposes the distal L-cells to incoming nutrients [144]. Similar effects are elicited by α-glucosidase
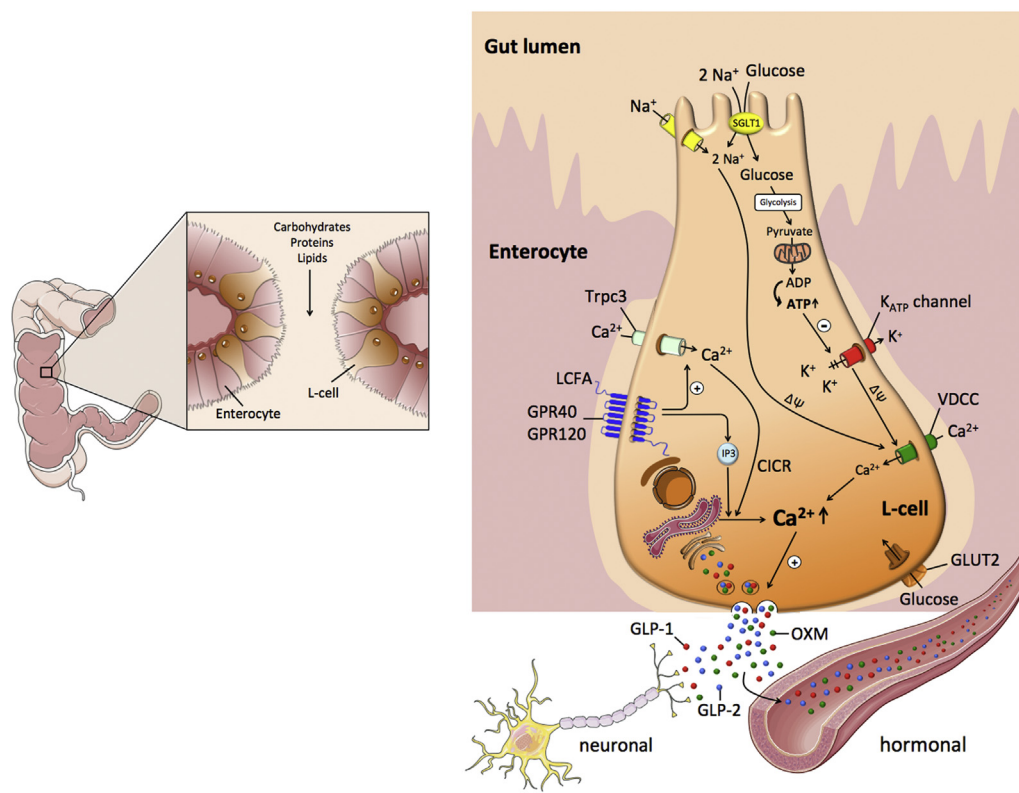
MOLECULAR
METABOLISM

Figure 3: Schematic on the nutrient-induced stimulation of GLP-1 secretion in the L-cell. CICR: calcium-induced calcium release; LCFA: long-chain fatty acids, GLUT2: glucose transporter 2; GLP-1: glucagon-like peptide-1; GLP-2: glucagon-like peptide-2; OXM: oxyntomodulin; Trpc3: transient receptor potential channel 3; VDCC: voltage-dependent calcium channel; SGLT1: sodium/glucose co-transporter 1. For further explanations, please see text.

inhibitors, which prevent digestion of starch and oligosaccharides, thus moving the unprocessed nutrients to more distal parts of the gut. In some studies, depending on subject characteristics and nutrient load, GLP-1 responses were augmented by acarbose or voglibose (α-glucosidase inhibitors) [217,218]. The commonly observed increase in GLP-1 after bariatric surgery [211—216] is considered a causal factor leading to increased circulating levels of insulin after the surgery. In line with this notion, blockade of GLP-1R with exendin-9 has been demonstrated to normalize post-bariatric hyperinsulinemia and alleviate resulting hypoglycemia after RYGB [219] or gastrectomy [217]. Similarly, blockade of GLP-1R with an antagonistic monoclonal antibody demonstrated an important contribution of enhanced GLP-1 secretion to the improved insulin secretion and glucose handling after vertical sleeve gastrectomy (VSG) in preoperatively lean mice [144]. The commonly observed rise in GLP-1 following gastric bypass surgery is also consistent with the robust responses observed in humans upon direct introduction of nutrients, in amounts corresponding to the "physiological malabsorption", by distal ileal intubation [210]. Thus, increased circulating GLP-1 is invariably reported after bariatric surgery [211—216] and after surgical repositioning of the distal gut (so-called ileal interposition), both of which rapidly expose the L-cells to incoming incompletely digested nutrients [144].

Enhanced GLP-1 secretion has been suggested to be a major mechanism underlying enhanced insulin secretion after bariatric surgery because blockade of GLP-1 action by treatment with the pharmacological antagonist exendin-9 decreased insulin secretion and ameliorated post-surgical glucose handling in both mice and humans [144]. Nonetheless, a primary role of GLP-1 in post-surgical improvement of

metabolism is controversial. Studies in GLP-1R KO mice documented that obese mice exhibit comparable weight loss and improved glucose metabolism to wildtype controls following VSG [220]. Failure of GLP-1R KO to attenuate the beneficial effects of bariatric surgery on body weight may be related to the observation that the body weight decrease in mice after RYGB may reflect enhanced energy expenditure, whereas in humans and in rats a reduction in food intake is more important [221]. However, sleeve gastrectomy differs markedly from gastric bypass with faster systemic appearance of ingested glucose and higher secretion of insulin, GLP-1, PYY, CCK, and ghrelin after RGYB [222]. Perhaps analogously, rodent experiments have found that GLP-1R signaling is not required for the weight-reducing effect of Roux-en-Y Gastric Bypass (RYGB) [223]. Furthermore, GLP-1 inhibition by exendin (9—39) or administration of a DPP-4 inhibitor does not affect food intake in humans following RYGB [224]. However, as demonstrated in that same report, concomitant administration of exendin9-39 and a DPP-4 inhibitor increased food intake by ∼20%, suggesting that GLP-1, when potentially acting in concert with other gut hormones, might have an important role in the metabolic benefits achieved by bariatric surgery [224]. Consistent with this, treatment of RYGB patients with exendin (9—39) increases the fMRI response to images of food in the caudate nucleus, and in the insula in the human brain in response to consuming palatable food [225].

The improved glucose metabolism is mainly due to three closely interacting factors: 1) rapid absorption of glucose from the intestine giving rise to high post-prandial glucose responses, 2) a consequent exaggerated secretion of GLP-1 that act on the β-cells, and 3) radically improved hepatic and subsequently peripheral insulin sensitivity [226].

Review

Like pancreatic α-cells, the intestinal L-cells secrete PGDPs simultaneously and in equimolar concentrations. An exception is glicentin and its cleavage product oxyntomodulin, the concentrations of which need to be combined to match the amount of secreted GLP-1 [41,53]. Meal-induced GLP-1 secretion has been demonstrated in numerous species including mice [227], rats [228−232], dogs [233−235] and humans [236−239]. An important consideration when analyzing meal-induced GLP-1 secretion is whether GLP-1 was measured in systemic or portal vein blood, whether the samples were taken in relation to oral meals or rather to intragastric infusion of liquid (or semiliquid) meals, and whether total or active GLP-1 was measured. As comprehensively discussed previously [240], an estimate of up to 75% of active GLP-1 is already degraded in the gut, and, from the amount of GLP-1 reaching the liver, another 50% is degraded before reaching the systemic circulation [183,241]. Only 10−15% of active GLP-1 is believed to reach the pancreas via the circulation [185,191,241]. As demonstrated in anesthetized pigs, blood concentrations of total and active GLP-1 progressively decrease with increasing distance from the site of secretion [241]. Levels of total and active GLP-1 are thus highest in the portal vein and lowest in the peripheral venous system [241]. In anesthetized pigs, baseline levels of total GLP-1 in the portal vein are in the range of ∼30 pmol/l and increase up to 150 pmol/l upon treatment with neuromedin C, which is a known stimulator of GLP-1 secretion [241]. Baseline portal vein levels of active GLP-1 are ∼10 pmol/l and increase up to ∼40 pmol/l upon treatment with neuromedin C [241].

Systemic plasma levels of total C-terminally amidated GLP-1 correlate with plasma insulin and thus are low during fasting and increased in response to an oral meal [236]. In humans, fasting systemic plasma concentrations of "total" GLP-1 (including metabolites generated by DPP-4-mediated degradation) are typically in the range of 5−10 pmol/L and can increase up to 40 pmol/L in response to a meal [236]. Plasma systemic concentrations of "intact", biologically active GLP-1 are much lower (fasting: < 2 pmol/l, peak post-nutrient concentrations 5−10 pmol/l) [240,242]. The amount of GLP-1 secretion is affected by the size and composition of a meal [237]. In healthy human volunteers, a 520 kcal oral meal induced a greater systemic increase of plasma systemic total and active GLP-1 than a 260 kcal meal [237]. GLP-1 levels are detectable during fasting, indicating that GLP-1 is tonically secreted into the general circulation in basal conditions [243]. In rats however, there is also evidence for a "preprandial" cephalic-phase GLP-1 reflex [244,245], and the muscarinic cholinergic antagonist atropine reduces the GLP-1 response to an oral glucose load in humans [246].

Nutrients stimulating GLP-1 secretion include the metabolizable monosaccharides that include glucose, fructose, and galactose [239,247−250] as well as non-metabolizable monosaccharides such as methyl-α-glucopyranoside [247], long-chain fatty acids [232,251−253], proteins [228,250,254−257], and certain amino acids [258−260]. In healthy humans, ingestion of carbohydrates or proteins elicits a rapid increase in circulating GLP-1 with a peak 30−60 min following nutrient intake, whereas ingestion of lipids elicits a somewhat later but more prolonged (>120 min) increase [238,239]. In rats, portal vein levels of total GLP-1 peak around 15 min after intragastric infusion of a liquid meal and return to baseline levels after 90−120 min [231]. In rats eating a spontaneous chow meal, increased portal vein but not systemic levels of active GLP-1 are detectable [261]. There is controversy as to whether glucose-induced GLP-1 secretion is disturbed in patients with type-2 diabetes. A study in ∼1,500 Danish subjects suggested that the GLP-1 response to oral glucose is reduced in patients with prediabetes or type-2 diabetes [262]. In contrast, a meta-analysis of 22 clinical studies revealed no difference in glucose-stimulated GLP-1 secretion between patients with type-2 diabetes and non-diabetic controls [263,264].

6.2. GLP-1 secretion in response to monosaccharides and other carbohydrates

The cellular mechanisms underlying glucose-stimulation of GLP-1 secretion from L-cells are, at least in part, similar to the stimulation of insulin secretion in the islets. In enteroendocrine GLUTag cells, glucose, and fructose dose-dependently increase GLP-1 secretion through closure of ATP-sensitive K_{ATP} channels and subsequent membrane depolarization (Figure 3) [247,249,265]. Glucose-induced membrane depolarization entails opening of voltage-dependent Ca^{2+} (VDC) channels, and the resulting Ca^{2+} influx then triggers vesicular exocytosis and secretion of GLP-1 into the circulation (Figure 3) [266]. Underlining the role of the K_{ATP} channels in mediating this process, glucose-stimulated Ca^{2+} entry and GLP-1 secretion are mimicked upon treatment of GLUTag cells with the K_{ATP} channel inhibitor tolbutamide [265]. While the importance of K_{ATP} channel activity in mediating GLP-1 release has been confirmed in vitro, its relevance for GLP-1 secretion in vivo is less clear. While sulphonylureas potently promote insulin secretion in type-2 diabetic patients via inhibition of K_{ATP} channel activity [267−270], there is no clear evidence that sulphonylureas affect GLP-1 secretion in humans (as reviewed in [266]). However, the Kir6.2/SUR1 channel complex of the K_{ATP} channel is present in human L-cells [271] and K_{ATP} channel subunits and glucokinase are highly expressed in murine L-cell populations [266]. In summary, while L-cell depolarization is crucial for GLP-1 secretion, the role of the Kir6.2/SUR1 channel complex of the K_{ATP} channels for mediating this process in vivo warrants clarification.

Monosaccharides demonstrated to stimulate GLP-1 secretion include glucose, galactose, and fructose [239,247]. Low concentrations of glucose or methyl-α-glucopyranoside stimulate L-cell electrical activity and promote GLP-1 secretion via sodium-glucose cotransporter (SGLT1)-dependent induction of small inward currents (Figure 3) [247]. Preventing luminal glucose absorption by blockade of SGLT1 reduces GLP-1 secretion in the isolated perfused canine ileum [248] and in the rat ileum [178] and impairs glucose-stimulation of GLP-1 release in GLUTag cells [247]. Notably, the glucose transporter-2 (GLUT2) has been implicated in glucose-stimulated GLP-1 secretion, as demonstrated by an impaired GLP-1 response to oral glucose in mice deficient for GLUT2, and this is accompanied by reduced glucose-stimulated insulin secretion and impaired glucose tolerance [272]. However, pharmacological inhibition of active, sodium-coupled glucose transport impaired glucose-stimulated GLP-1 secretion in vitro, whereas inhibition of facilitative GLUT-mediated glucose transport was without effect [273]. In summary, glucose uptake into the L-cells seems to be mediated via both, GLUT2 and SGLT1 (Figure 3) [274−276], with the electrogenic SGLT1 mediated uptake being of particular importance for stimulus secretion coupling. Downstream of glucose-mediated membrane depolarization, vesicular exocytosis of GLP-1 is orchestrated in a Ca^{2+} dependent manner involving a cellular machinery like that in β-cells [277,278]. Fructose stimulation of GLP-1 secretion has been demonstrated in rats, mice, humans, and GLUTag cells [249,279]. When given orally, fructose is a far less potent GLP-1 secretagogue relative to an isocaloric load of glucose [249]. Similar findings are reported in humans upon intragastric infusion of glucose and fructose at doses that are matched for sweetness [279].

A possible role of intestinal sweet-taste receptors in glucose-stimulated GLP-1 secretion remains uncertain. In isolated murine

and human L-cell cultures, glucose, and the artificial sweetener sucralose, each stimulates GLP-1 secretion with diminished glucose stimulation of GLP-1 secretion in mice lacking α-gustducin, an integral sweet-taste receptor element [280]. In contrast, there is no effect of sucralose on GLP-1 secretion in primary L-cell cultures in other reports [281,282], and infusion of artificial sweeteners does not affect glucose-stimulated GLP-1 secretion in healthy human volunteers [283,284].

6.3. GLP-1 secretion in response to dietary lipids

In addition to sensing carbohydrates and glucose, L-cells, like other enteroendocrine cells, sense dietary lipids and protein (see next section) through specific cell-surface receptors, which bind metabolites of the dietary lipids and proteins [285] (Figure 4). This occurs mainly at the basolateral side of the L-cell, i.e. after absorption of the metabolites [285]. In the case of dietary triglycerides, the L-cell responds not only to the free fatty acids but also to the other major metabolite product, 2-monoacyl glycerol (2-MAG), which appears to act in synergy [286].

Cell lines commonly used to study mechanisms of GLP-1 secretion are GLUTag, STC-1 and human colorectal adenocarcinoma-derived NCI-H716 cells. These cell lines are far from optimal models, as they, for example, differ in their secretory repertoire; some secreting peptides that are not classical L-cell products, such as GIP, glucagon, and somatostatin, and GLUTag cells lack PYY [287].

Fatty acid induction of GLP-1 secretion has been demonstrated *in vitro* using murine enteroendocrine STC-1 cells [251] and human intestinal NCI-H716 cells [251,253] and *in vivo* by direct administration of lipids into the duodenum [288,289] or ileum [210]. Dose-dependent fatty acid-induced GLP-1 secretion is evident with α-linolenic acid (C18:3), docosahexanoic acid (C22:6), and palmitoleic acid (C16:1), oleic acid (C18:1), stearic acid (C18:0), and octanoic acid (C8:0) [251] are less

effective. In humans, unsaturated are more effective than saturated fatty acids [290,291].

Induction of GLP-1 secretion by FFA is highly dependent on the cytosolic Ca^{2+} concentration. Treatment of STC-1, GLUTag, or NCI-H716 cells with long-chain fatty acids potently increases intracellular Ca^{2+} [253,292]. The FFA-induced rise in intracellular Ca^{2+} is substantially reduced when cells are cultured in a Ca^{2+} free medium, and is abolished upon treatment of cells with the Ca^{2+} channel inhibitor nicardipine [292] or when using BSA (which binds fatty acids) [251]. Collectively, these data suggest that FFAs increase intracellular Ca^{2+} by stimulating the influx of Ca^{2+} via the cell-surface (most likely L-type) Ca^{2+} channels [292]. Treatment of NCI-H716 or STC-1 cells with the Ca^{2+} ionophore ionomycin or with phorbol myristate acetate (PMA) increases cytosolic Ca^{2+} levels and stimulates GLP-1 secretion in a dose-dependent manner [251,253]. The ionomycin-induced increase in Ca^{2+} influx and GLP-1 secretion is completely abolished upon treatment of cells with the Ca^{2+} chelator EGTA [251]. In summary, compelling evidence indicates that FFA increase GLP-1 secretion by stimulating extracellular Ca^{2+} influx via cell-surface Ca^{2+} channels.

Receptors implicated in FFA regulation of GLP-1 secretion include GPR120 (FFAR4) and GPR40 (FFAR1), both of which are activated by long-chain fatty acids. GPR120 was previously reported to be co-localized with GLP-1 in colonic enteroendocrine cells [251]. This could point to GPR120 being involved in the stimulation of GLP-1 by long chain FFAs. However, The GLP-1 response to oleic acid is unaltered in GPR120-deficient mice, and synthetic GPR120 agonists do not stimulate GLP-1 from primary cell cultures [286]. GPR40, the other long chain FFA receptor, is also highly expressed and the most enriched GPCR in L-cells [266,293]. In contrast to GPR120, the GLP-1 response to dietary fat is strongly reduced in GPR40 KO mice as compared to littermates [286]. Importantly, synthetic GPR40 agonists
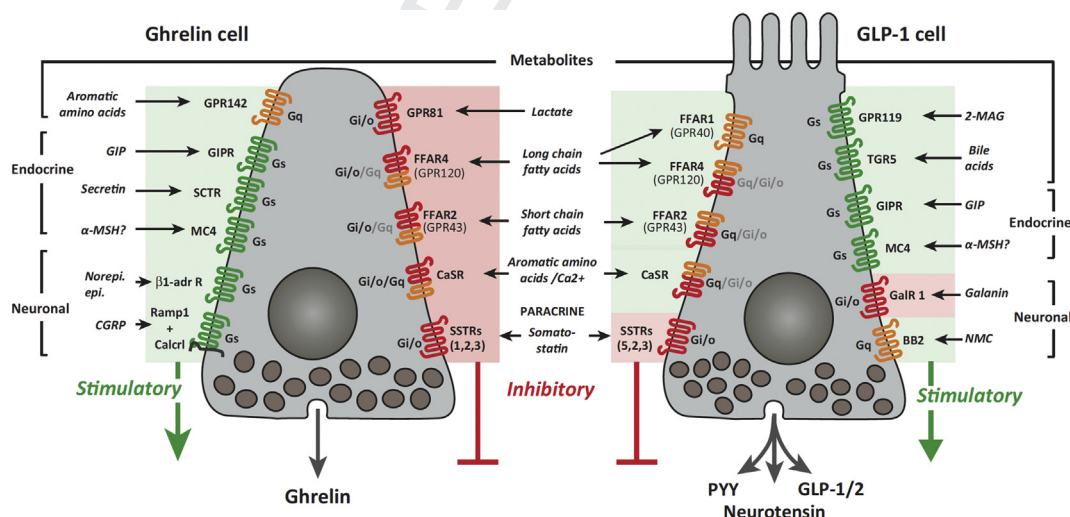

Figure 4: Schematic on the GPCR Repertoire Involved in Control of Hormone Secretion from Gastric cells expressing either ghrelin or GLP-1. Green or red color background color indicates stimulation (green) or inhibition (red) of hormone secretion upon receptor activation. The colors of the receptors represent G protein signaling potential via G/s (green), G/q (orange) or G/i/o (red). The type of G protein responsible for the effect on hormone secretion has not been determined for all the receptors; for those where it has not been described, we have indicated the most likely coupling, based on data from other cell studies. 2-MAG, 2- monoacyl glycerol; BB2, bombesin receptor 2; Calcrl, calcitonin receptor-like receptor; CaSR, calcium sensing receptor; CGRP, calcitonin gene-related peptide; FACS, fluorescence-activated cell sorting; FFAR, free fatty acid receptor; GalR1, galanin receptor 1; GIP, glucose-dependent insulinotropic peptide; GIPR, GIP receptor; GPR, G protein receptor; MC$, melanocortin 4; MSH, melanocyte-stimulating hormone; NMC, neuromedin C; PYY, peptide YY; Ramp1, receptor activity modifying protein 1; SCTR, secretin receptor T; SSTR, somatostatin receptor; TGR5, bile acid receptor.

Review

are efficacious GLP-1 secretagogues both *in vitro* in primary cultures, in perfused intestines and *in vivo* when studied in mice [252,294,295]. Treatment of mice with GPR40 agonists [296,297] has been reported to decrease body weight and food intake in DIO mice, effects notably absent in mice deficient for either GPR40 or GLP-1R [296].

Accordingly, long chain fatty acids stimulate GLP-1 secretion mainly through GPR40. However, the other main metabolite from dietary tri-glycerides, 2-MAG, is also a very powerful GLP-1 secretagogue acting through GPR119 [298,299]. Importantly, the GLP-1 response to dietary fat is vastly reduced not only in GPR40 KO animals but also in GPR119 KO mice, and agonists for the Gq-coupled GPR40 act in synergy with the Gs-coupled GPR119 to robustly stimulate GLP-1 [286,300]. A receptor-independent stimulation of GLP-1 release from GLUTag cells has been reported for oleic acid, which stimulates GLP-1 release by uncoupling oxidative phosphorylation and, hence, indirectly stimulating glycolysis with the resulting activation of the mechanism alluded to above [301].

6.4. GLP-1 secretion by proteins/amino acids

Protein and amino acid stimulation of GLP-1 secretion has been demonstrated in murine primary colonic L-cell cultures [260,302], in GLUTag cells [256,259], in human NCI-H716 cells [251,303] and in the isolated perfused rat ileum or colon [250,304] as well as *in vivo* in mice [305], rats [256,306], and humans [307,308]. In healthy human volunteers, a diet with 30% kcal from protein (40% carbohydrates, 30% fat) causes greater GLP-1 secretion than a diet with 10% kcal protein (60% carbohydrates, 30% fat) [308]. Individual amino acids stimulating GLP-1 secretion include glutamine, asparagine, phenylalanine, and glycine, with glutamine and glycine being the most potent [258–260]. When orally administered, glutamine also increases circulating GLP-1 and insulin in lean, obese and type-2 diabetic individuals [307]. In human NCI-H716 cells, stimulation of GLP-1 secretion was also demonstrated for leucine, isoleucine, valine, skimmed milk, casein, and whey [303]. L-Arginine, a potent insulin secretagogue [309], also stimulates GLP-1 release from isolated rat intestine, and, when given orally, augments GLP-1 and insulin levels and improves glucose tolerance in mice, effects that are absent in GLP-1R KO mice [305].

Meat hydrolysate stimulates GLP-1 secretion from NCI-H716 cells, an effect that is not related to changes in proglucagon expression [253]. This stimulatory effect can be blocked by pretreatment with the p38 inhibitor SB203580, the PI3 kinase inhibitor wortmannin or the MEK1/2 inhibitor U0126 [310]. The corn protein zein stimulates GLP-1 secretion in GLUTag cells and in the small intestine of anesthetized rats [256], and it stimulates GLP-1 secretion when administered either orally [228] or directly into the ileum [257]. Intraluminal administration of peptones stimulate GLP-1 secretion in the isolated perfused rat ileum [250] but not upon ileal perfusion in healthy human volunteers [210]. Pectin stimulates GLP-1 secretion in the isolated perfused rat colon [304] but not in the isolated rat ileum [250]. The low molecular fraction of wheat protein hydrolysate (LWP) increases GLP-1 secretion in both GLUTag cells and when directly administered in rats [306]. In rats, the LWP-induced GLP-1 secretion further improves glucose tolerance and enhances insulin secretion, an effect that is blocked by pre-administration of the GLP-1R antagonist exendin (9–39) [306]. Protein-stimulation of GLP-1 secretion has also been demonstrated in humans, with similar GLP-1 responses upon uptake of whey, casein, gluten or cod protein [311–313]. Protein-induction of GLP-1 secretion seems to be dose-dependent, as demonstrated by uptake of isocaloric diets comprising 14%, 25%, or 50% of energy coming from proteins [314].

The molecular mechanisms underlying protein stimulation of GLP-1 secretion include activation of Ca^{2+}/calmodulin-dependent kinase II [306]. Substantial evidence supports that peptide-mediated GLP-1 secretion is a Ca^{2+} sensitive process and involves L-cell signaling via the Ca^{2+} sensing receptor (CaSR) and the peptide transporter 1 (PEPT1) [302]. Consistent with this, glycine-sarcosine (Gly-Sar) stim-ulation of GLP-1 secretion from purified murine L-cell cultures is blocked in the absence of extracellular Ca^{2+} and is inhibited upon treatment with the L-type Ca^{2+}-channel blocker nifedipine [302]. Oligopeptide stimulation of GLP-1 release is impaired upon treatment of L-cell cultures with a CaSR antagonist and is ameliorated in mice deficient for the peptide transporter 1 (PEPT1) [302]. Aromatic amino acids such as phenylalanine, however, also interact with GPR142 [315].

6.5. GLP-1 secretion in response to endocrine factors

6.5.1. Endocrine regulation of intestinal GLP-1 secretion

The intestinal distribution of L-cells, with high abundance in the distal gut and low abundance in the proximal gut, argues for the existence of a proximal-to-distal coordinating loop in which neuronal and/or endocrine factors arising in the upper intestine affect L-cell GLP-1 secretion in the distal region. While such a proximal-distal loop might indeed exist, it cannot be ruled out that while fewer in number, L-cells in the upper intestine are sufficient for the rapid induction of GLP-1 secretion following nutrient intake [316–318]. Nonetheless, the presumed loop (if it exists) would likely be important for the early postprandial phase at a time when the L-cells of the distal gut are not yet in direct contact with luminal nutrients. Supporting such neuronal/endocrine regulation of GLP-1 secretion, the L-cells are in close proximity to both enteric neurons and the intestinal microvasculature [191,319]. Possible neuroendocrine regulation of GLP-1 secretion is supported by studies in rodents in which nutrient flow to the distal intestine is prevented, precluding direct L-cell contact in this part of the intestine to luminal nutrients, [289,320]. Administration of glucose or fat directly into the duodenum of such rodents rapidly stimulates L-cell GLP-1 secretion, with a magnitude comparable to that occurring when nutrients are directly placed into the ileum [289,320]. Because the L-cells co-secrete the PGDPs, factors stimulating GLP-2 or oxy-ntomodulin are also natural secretagogues of GLP-1. Neuronal/endo-crine factors affecting the intestinal release of PGDPs in some species include GIP, acetylcholine, gastrin-releasing peptide (GRP), insulin, somatostatin, and ghrelin [320–322].

In rodents, a biphasic secretion of GLP-1 has been observed; this suggests a rapid phase of GLP-1 secretion caused by direct stimulation of L-cells in the upper GI tract and a second phase potentially caused by signals from the upper gut reaching the lower small and perhaps even the large intestine [323,324]. GIP and the intramural intestinal autonomous nervous system have been suggested as signaling pathways [323]. Several lines of evidence support a role for GIP in GLP-1 secretion. GIP stimulation of GLP-1 secretion, however, seems to be highly species-specific. Levels of GIP expression (in K-cells) are greatest in the proximal gut, and circulating levels of GIP rapidly in-crease upon food intake [236,325,326] or when nutrients are placed directly into the duodenum [289,323,327]. Secretion of preproglucagon-derived peptides is stimulated in rats and in primary rat L-cell cultures upon treatment with GIP [289,323,327]. In rats, induction of gut glucagon-like immunoreactivity induced by either lipids or by physiological concentrations of GIP can be blocked by subdiaphragmatic vagotomy, suggesting that in rats GIP regulation of

GLP-1 secretion requires either afferent or efferent signal transmission via the vagus [327]. The importance of such a proximal-to-distal GIP-GLP-1 axis for human physiology is, however, questionable. In healthy and type-2 diabetic humans, it has been consistently demonstrated, that GIP, even in rather high (supraphysiological) doses, does not lead to GLP-1 secretion [328—330]. In humans, GLP-1 secretion in response to duodenal glucose delivery does not become robustly stimulated until the delivery rate overcomes the absorptive capacity of the duodenum, i.e. until such time as non-absorbed glucose reaches the jejunum and beyond. In contrast, GIP secretion is stimulated by very low gastric glucose delivery rates, consistent with the higher abundance of GIP-producing K-cells compared to GLP-1-producing L-cells in the proximal intestine [331]. Direct infusion of glucose into the duodenum at a rate that ensured ~ total absorption close to the infusion site (2 kcal/min) triggered robust GIP, but not GLP-1 secretion, whereas both hormones were released when glucose was directly delivered into the ileum [332]. Another gut peptide that has been implicated in a proximal-to-distal loop to stimulate GLP-1 secretion is CCK [333]. However, the concentrations of both GIP and CCK needed to stimulate GLP-1 secretion are not normally reached under physiological conditions [334].

Neurotransmitters expressed in vagal and enteric neurons, including acetylcholine and GRP, increase GLP-1 secretion, supporting the possibility of a proximal-distal neuroendocrine loop [335] without the need of supraphysiological plasma concentrations of proximally-secreted gut peptides. Receptors for acetylcholine, including the muscarinic receptors M1, M2, and M3, are expressed in rat L-cells [319] and human NCI-H716 cells [336]. Treatment of rats with the nonspecific muscarinic receptor antagonist atropine or with the M1 selective antagonist pirenzipine, but not treatment with M2 or M3-selective antagonists blunts lipid-induced GLP-1 secretion [319,335]. In human NCI-H716 cells, GLP-1 secretion is stimulated by betha-nechol, a nonselective muscarinic agonist, while pretreatment with pirenzipine or the M2 antagonist gallamine inhibits bethanechol-induction of GLP-1 secretion [336]. Acetylcholine also stimulates GLP-1 secretion in the perfused porcine ileum, and this effect can be blocked by co-infusion of atropine [337]. Albeit with notable physiological challenges (atropine powerfully inhibits GI motility), infusion of atropine also blunts nutrient induced GLP-1 secretion in healthy human volunteers [246]. Together, these data suggest that M1 and M2 muscarinic receptors are implicated in human L-cell GLP-1 secretion. Interestingly, in rat ileum preparations, administration of atropine was not able to block GIP-induced GLP-1 secretion [335]. In the isolated perfused porcine ileum, GLP-1 secretion is inhibited by electrical nerve stimulation or by administration of norepinephrine, effects that can be blocked by co-infusion of the nonselective α-adrenergic receptor antagonist phentolamine [337]. While norepinephrine seemingly inhibits GLP-1 secretion via its action on α-adrenergic receptors, its secretion is stimulated by isoproterenol, and this effect can be blocked by co-infusion of the β-adrenergic receptor antagonist propanolol [337]. These data collectively suggest that intestinal GLP-1 secretion is stimulated by cholinergic and β-adrenergic receptor signaling and inhibited by activation of α-adrenergic receptors.

GRP is produced and released by GRPergic neurons of the enteric nervous system [338]. In rats, infusion of GRP stimulates the secretion of GLP-1 while administration of the GRP antagonist BW10 blocks GLP-1 secretion when fat is directly administered into the duodenum [339]. GRP stimulation of GLP-1 secretion has also been demonstrated for rat L-cell cultures and in preparations of rat ileum [323,335]. Notably, GRP regulation of glucose handling is not fully dependent on GLP-1 signaling, because GRP also directly stimulates insulin secretion in the isolated

perfused dog pancreas [340] and further delays gastric emptying [341,342]. Nonetheless, mice deficient for GRP have impaired glucose tolerance, reduced first-phase insulin secretion and impaired GLP-1 secretion in response to an oral glucose challenge [343].

In summary, there are several mechanisms that may contribute to the rapid increase in GLP-1 secretion following nutrient intake. Nutrient-induced GLP-1 secretion can occur from L-cells located in the proximal small intestine with induction of GLP-1 secretion as early as digested nutrients leave the pylorus. The glucose concentration after a meal may exceed the absorptive capacity in the proximal intestine so that the ingested glucose rapidly reaches the more distally located L-cells. Neuroendocrine reflexes may also trigger GLP-1 secretion in addition to direct nutrient-induced stimulation of L-cell GLP-1 secretion [344]. Current data suggest that when chyme enters the duodenum it triggers GIP release in the proximal gut. The local increase in GIP stimulates vagal afferent transmission followed by activation of vagal efferents and enteric neurons that release acetylcholine and/or GRP to stimulate GLP-1 release from the distal gut. When the nutrients subsequently reach the distal gut, direct contact with the L-cells then triggers additional GLP-1 secretion into the circulation (as also reviewed in [320]).

Other factors influencing GLP-1 secretion include activation of the olfactory receptor OR51E1 using nonanoic acid, which stimulates secretion of GLP-1 and PYY in human and rodent enteroendocrine L-cells [345]. More recently, ghrelin was identified to stimulate GLP-1 secretion in murine and human L-cell cultures [322]. In mice, peripheral administration of ghrelin further enhances glucose-stimulated GLP-1 secretion and improves glucose tolerance, an effect that is blocked by pre-administration of the ghrelin receptor antagonist D-Lys GHRP6 and that is absent in GLP-1R KO mice [322].

6.5.2. Endocrine regulation of central GLP-1 secretion

As discussed above, in addition to enteroendocrine L-cells, GLP-1 is also produced in a discrete set of non-TH-positive neurons in the caudal portions of the NTS [86,148,346—348], and these hindbrain GCG + positive neurons are the primary source of endogenous brain GLP-1 [90]. Either peripheral administration of leptin [349] or gastric balloon distention [350] acutely activates GLP-1-producing neurons in the NTS, as assessed by cFos immunoreactivity. Direct electrical stimulation of the NTS evokes glutamatergic excitatory post-synaptic currents (EPSCs) in GCG + positive neurons [351]. Generation of mice that express eYFP under control of the *Gcg* promoter has enabled the isolation and characterization of NTS GCG + neurons in *ex vivo* tissue slices [351]. Electrophysiological whole-cell voltage- and current-clamp recordings in horizontal or coronal brainstem slices has revealed a rapid leptin-induced depolarization of these NTS GCG + neurons, thus confirming the ability of leptin to directly stimulate central GLP-1 secretion [351]. Of note, the hindbrain GCG + neurons lack the GLP-1 receptor such that they cannot be directly activated by peripherally-derived GLP-1 [351]. In addition, neither electrophysiological administration of PYY, melanotan II, nor ghrelin stimulates these neurons in isolated NTS brain slices [351]. In contrast, leptin [351], CCK, and epinephrine [352] stimulate Gcg neurons. In the NTS, neurons expressing GLP-1 also express the leptin receptor [351,353]. Electrical stimulation of the solitary tract indicates that PPG neurons in the NTS are second-order neurons that receive direct input from vagal afferents. Thus, peripheral endocrine signals, such leptin or GLP-1, can via activation of vagal afferents trigger central activation of PPG neurons in the NTS [351].

CCK-induced firing of the NTS GCG + neurons can be blocked by treatment with the glutamate receptor antagonist DNQX or by inhibition

Review

of α1-adrenergic signaling [352]. Consistent with these findings, peripherally administered CCK induces cFos immunoreactivity in GLP-1-producing neurons of the hindbrain vagal complex of the NTS [354], and surgical vagal deafferentation reduces CCK-induced NTS neuronal cFos activation by approximately 50% [355]. Thus, these neurons are able to sense and respond to a variety of peripheral signals that help to regulate both short and long-term energy balance. Indeed, similar to chronic blockade of CNS GLP-1R, viral knockdown of GLP− expressing neurons in rats increases body mass, and specifically body adiposity [356]. In a recent report, chemogenetic stimulation of Gcg neurons reduced food intake without conditioning avoidance, and this occurred when the animals were fed or fasted or were fed chow or HFD [357]. On the other hand, acute chemogenetic inhibition of these neurons did not increase ad lib feeding but did increase refeeding after a fast and blocked stress-induced hypophagia [90]. In summary, the glutamatergic GLP-1-producing neurons in the NTS are activated by multiple peripheral signals and regulate many aspects of feeding behavior. This CNS GLP-1 system does not seem to be activated by peripherally-secreted (endogenous) GLP-1 and therefore may be distinct from the peripheral GLP-1 system.

7. THE GLUCAGON RECEPTOR FAMILY

GLP-1, GLP-2, glucagon, GIP, secretin, and growth hormone-releasing hormone (GHRH) belong to a group of structurally related peptides that promote their biological action via binding to structurally similar G protein-coupled receptors (GPCRs) of the class B family [358,359]. All members of this family are seven transmembrane $G\alpha_s$-coupled receptors that increase levels of cAMP through activation of adenylate cyclase [359]. Each receptor of this family is concisely named based on its single and unique endogenous ligand (GLP-1R, GLP-2R, GCGR, GIPR, SCTR, and GHRHR). Under physiological conditions, most studies report no meaningful cross-reactivity among the peptide ligands and the receptors of this family [359,360].

Cloning of rat and human pancreatic GLP-1R cDNA documented that ligand-induced activation of a single unique GLP-1R increases intracellular levels of cAMP and also that GLP-1, but not glucagon, GIP, VIP, or secretin, activates GLP-1R [360−364]. In the pancreas, glucagon has physiologically relevant cross-reactivity with GLP-1R, with an EC_{50} of 36.4 ± 0.22 nM, but there is no affinity of GLP-1 to the glucagon receptor [365]. The interaction of glucagon with GLP-1R is important for insulin secretion in the β-cells [365]. Cloning of the human pancreatic GLP-1R cDNA was also used to demonstrate comparable binding affinity of exendin-4 and exendin (9−39) to human GLP-1R [363], and consolidated the work of Jean-Pierre Raufman and John Eng that identified exendin-4 as a GLP-1 paralog [366] and exendin (9−39) as a GLP-1R antagonist [367].

7.1. Tissue distribution of GLP-1R

The presence of GLP-1R was first demonstrated in rat insulinoma RINm5F [368], RIN5AH [369], and RIN1046-38 cells [82] using cAMP accumulation assays and radioligand binding. Subsequent studies then confirmed the presence of GLP-1R in these and other insulinoma cell lines [369−376] as well as in somatostatin-secreting cells [370,375] and in islets isolated from rats [377] and humans [378].

Expression of GLP-1R was also demonstrated in the rat lung [379] as well as in the human brain, kidney, stomach and heart, with no expression of GLP-1R in liver, skeletal muscle or adipose tissue [380,381] Although early efforts [380,381] failed to consistently detect the GLP-1 receptor in adipose tissue, more recent studies have

unequivocally identified the GLP-1R expressed in adipocytes. These recent studies include the finding of the receptor in differentiating mouse 3T3-L1 pre-adipocytes [382−384], adipocytes formed from human bone marrow-derived mesenchymal stem cells [385], human epicardial fat [386], and in human visceral white adipose tissue [387]. Nonetheless, GLP-1 and liraglutide induce adipocyte formation in vitro and in vivo, and adipogenesis is decreased in preadipocytes lacking GLP-1R [384]. Autoradiography studies identified binding of [125I]GLP-1 and [125I][Tyr39]exendin-4 in the rat lateral septum, subfornical regions, thalamus, hypothalamus, interpenduncular nucleus, posterodorsal tegmental nucleus, area postrema, inferior olive, and NTS [388]. Similar findings on the central distribution of GLP-1R mRNA in rats have been reported using S35-UTP-labeled probes complementary to the PPG and GLP-1R mRNA [347]. In non-human primates, the highest GLP-1 immunoreactivity has been demonstrated in various nuclei of the hypothalamus, the area postrema, the NTS, and the dorsal motor nucleus of the vagus [389]. In the human brain, expression of GLP-1R mRNA has been reported in the cerebral cortex, hypothalamus, hippocampus, thalamus, caudate-putamen, and globus pallidum [390].

A general caveat when analyzing the tissue distribution of the GLP-1R protein is the lack of sufficiently selective and commercially available antibodies [391]. However, Novo Nordisk has recently developed monoclonal antibodies which, through targeting of the extracellular domain of GLP-1R, allow the detection of GLP-1R in human and rodent tissues with improved selectivity [392−394]. Using these antibodies, immunohistochemical studies in tissues from humans and non-human primates confirmed the presence of GLP-1R in the pancreas with broad expression in the β-cells, weaker abundance in the acinar cells, and no expression in the duct cells [392]. GLP-1R was further detected in the arterial walls of the kidney and lung, in heart myocytes of the sinoatrial node, and in the Brunner's gland of the duodenum [392]. In heart myocytes, expression of GLP-1R is restricted to the sinoatrial node and is not observed in other cardiomyocytes [392], which aligns with the scattered expression pattern of GLP-1R in the atrium but not elsewhere in the heart [395]. Low expression of GLP-1R occurred in parietal cells and smooth muscle cells of the stomach and in myenteric plexus neurons of the gut with no expression in the liver and thyroid gland [392]. In mice, GLP-1R-positive cells were identified in several regions of the telencephalon (olfactory bulb, amygdala, preoptic area, nucleus accumbens, septum), diencephalon (hypothalamic arcuate nucleus, paraventricular nucleus, dorsomedial nucleus, the lateral hypothalamic nucleus and the supraoptic nucleus) and regions of the mesencephalon [393]. In the medulla, GLP-1R was observed in the area postrema, NTS, and lateral reticular nucleus [393].

Complementary data on the distribution of GLP-1R have been reported using other validated techniques, including in situ hybridization and highly sensitive specific antisera. Further, studies of transgenic animals controlling the expression of green fluorescent protein (GFP) downstream of the GLP-1R promoter found GLP-1R promoter activity in pancreatic β- and δ-cells, vascular smooth muscle, cardiac atrium, the gastric antrum and pylorus, enteric neurons, and vagal and dorsal root ganglia [395]. In the brain, major GLP-1R-positive regions include the area postrema, ARC, VMH, and the ventrolateral medulla with lower expression in the PVH and NTS [395,396]. While GLP-1R is solidly expressed in the pancreatic β- and δ-cells, most studies report absence [397−399] or very restricted expression of GLP-1R in a small subset (<10%) of α-cells [395]. In a more recent study, detection of GLP-1R using a fluorescent GLP-1R antagonistic peptide label (Luxendin645) located GLP-1R on the cell surface of ∼18% of mouse α-cells [400].

7.2. Ligand-induced activation of GLP-1R

The rat and human GLP-1R protein comprises 463 amino acids with 90% sequence homology between these species [359,360,364]. GLP-1 binding to and activation of GLP-1R is a complex process that is comprehensively summarized in previous review articles [359,401]. As a class B GPCR, GLP-1R comprises seven transmembrane helices (TMH) interconnected by intracellular loops, with a C-terminal intracellular domain and a large (∼120 amino acid) N-terminal extracellular domain (ECD) [401]. Upon biosynthesis of the receptor in the endoplasmic reticulum (ER), the N-terminal ECD of GLP-1R (and of all other class-B GPCRs) contains a short leader sequence encoding for a signal peptide. This signal peptide is of crucial importance for translocation of the receptor across the ER as well as for trafficking of the receptor to the cell surface [359,401—404]. Underlining the importance of the signal peptide in receptor trafficking, blocking the signal peptide through site-directed mutagenesis causes retention of the receptor within the ER [404]. Following translocation of the receptor across the ER, the signal peptide is enzymatically cleaved by a peptidase, leaving behind the full length GLP-1R with the N-terminal helix at the beginning of the ECD and four β-strands forming two antiparallel sheets that are connected through three disulfide bonds between six cysteine (Cys) residues [401]. The disulfide bond between Cys^1 and Cys^3 connects the N-terminal α-helix of the receptor to the first β-sheet, while the disulfide bond between Cys^2 and Cys^5 connects two β-sheets and the disulfide bond between Cys^4 and Cys^6 holds the central β-sheets in proximity to the C-terminal domain of the receptor [401,405—409].

Several studies have aimed to identify the functional GLP-1 motifs orchestrating its interaction with GLP-1R [410—412]. Analysis of the crystal structure of GLP-1R linked to GLP-1 suggests that certain residues in the α-helical region of GLP-1 interact with residues in the N-terminal ECD of GLP-1R [407,409]. These data agree with reports demonstrating a crucial role of the GLP-1R ECD for recognition by GLP-1 [405,413—416]. Although these studies convincingly demonstrate the importance of the GLP-1R ECD for ligand binding, this interaction seems less important for ligand-induced activation of the receptor [401]. Accordingly, exendin (9—39) and exendin-4 have similar ligand binding affinities to the human GLP-1R, yet exendin (9—39) is not able to activate the receptor [363]. Furthermore, despite a low affinity to bind GLP-1R, [Ser(2)]exendin (1—9) is nonetheless able to activate the receptor with low potency [417]. Taken together, the ligand motifs required for receptor binding seem to differ from those motifs required for receptor activation. Indeed, for most (if not all) members of the glucagon receptor family, the activation of the receptor requires interaction of the N-terminal residues of the ligand with the transmembrane helices and extracellular loops of the receptor [401,418—420]. A two-domain model has emerged and suggests that GLP-1 binds via its α-helical and C-terminal motifs to the N-terminal ECD of GLP-1R followed by the activation of GLP-1R through binding of the N-terminal residues of GLP-1 with the transmembrane helices and extracellular loops of the receptor [401,421,422].

A model for ligand-induced receptor conformation has also been proposed based on comparative molecular simulations using GLP-1R bound to positive and negative allosteric modulators [423]. Based on this model, a negative allosteric modulator binds and deactivates GLP-1R through binding between the cleft of the GLP-1R helices VI and VII, thereby pushing helix VI to an inactive state that prevents association of the receptor with the G protein [423]. A positive allosteric modulator binds and activates GLP-1R through binding between helix V and VI, thereby creating an intercellular binding site for the G protein [423]. Similar conclusions have been reached by analysis of the GLP-1 bound receptor conformation using cryo-electron microscopy [424] and by analyzing the crystal structure of GLP-1R bound to truncated peptide agonist [425].

7.3. Ligand-induced biased GLP-1R agonism

Activation of GLP-1R stimulates formation of cAMP via G_s signaling, increases intracellular Ca^{2+} via the $G_{q/11}$ pathway, and promotes ERK1/2 signaling via recruitment of β-arrestin [426—431]. Despite binding to the same receptor, different GLP-1R ligands can engage selective pathways to elicit different cellular responses [427,431,432]. Such biased agonism is believed to have therapeutic value because certain ligands might accentuate specific signaling pathways that optimize therapeutic outcome. By assessing cAMP formation, Ca^{2+} accumulation and ERK1/2 phosphorylation, biased agonism was demonstrated for exendin-4 and oxyntomodulin, which, relative to GLP-1, have a bias for β-arrestin signaling [427,431,432]. In INS-1 cells, knockdown of β-arrestin attenuates GLP-1-stimulation of insulin secretion and decreases activation of ERK1/2 and CREB [429]. β-arrestin also promotes cell proliferation and survival via mechanisms that include activation of ERK1/2 [433]. Relative to GLP-1 and exendin-4, the biased GLP-1R agonist exendin P5 favors G protein coupled signaling over recruitment of β-arrestin [434]. In lean mice, exendin P5 improves glucose handling with greater potency than exendin-4 but has comparable efficacy to exendin-4 when analyzed more chronically in DIO and ob/ob mice [434]. *In vitro* comparison of receptor occupancy with ligand potency and efficacy demonstrated that the bias of exendin P5 results from enhanced efficacy in $G\alpha_s$-mediated cAMP signaling rather than a loss of β-arrestin recruitment [435]. Functional studies of GLP-1R signaling in combination with three-dimensional modeling of the ligand—receptor complex identified the GLP-1R ECD as the key molecular trigger of biased agonism. Based on this model, the ligand-specific response of the receptor is mediated by a unique receptor conformation/stabilization that results from the specific and individual contact of the ligand with the receptor [431]. When treated with either GLP-1 or exendin P5, the greatest difference in the ligand-bound receptor structure is observed in transmembrane 1 (TM1), the extracellular portions of TM6 and TM7, and the ECL3 conformation, suggesting that these ECDs modulate biased signal transduction [435].

7.4. GLP-1R desensitization and resensitization

Upon ligand binding, GPCRs located on the cell surface transduce the extracellular signal to the interior of the cell. At some point, the ligand-induced receptor activation has to be terminated, and the sensitivity of the receptor to be activated by its ligand has to be restored. Data related to the molecular mechanisms leading to termination of GLP-1R activity (desensitization) and subsequent GLP-1R resensitization are incompletely understood and partially conflicting.

Desensitization of GPCRs is generally achieved by two families of serine/threonine kinases, the second-messenger-dependent protein kinases and the receptor-specific G protein-coupled receptor kinases (GRKs) [436,437]. After ligand-induced receptor activation, these kinases phosphorylate the receptor. This induces intracellular recruitment of arrestin, which then binds to the GPCR and uncouples the receptor from its heterotrimeric G proteins [436,437]. Another potential mode of receptor deactivation is the translocation of the ligand—receptor complex to intracellular compartments (endosomes), such that the now intracellular receptor is physically dissociated from the membrane [436,437]. While such ligand-induced receptor internalization has been demonstrated for many GPCRs (including GLP-1R), its importance for receptor deactivation is controversial and is potentially receptor specific. While some studies support a role of receptor

Review

internalization in ligand-induced receptor desensitization [438,439], no such role has been demonstrated for the β2 adrenergic receptor (β2-ADR) [440–443] or the receptors for angiotensin$_{1A}$ [444], D1 dopamine [445], m2mACh [446], neurokinin 1 [447], histamine H2 [448], secretin [449], or GLP-1 [450–452]. However, although it may not be relevant for receptor inactivation, receptor internalization is nevertheless important for receptor resensitization. A commonly proposed model is that the phosphorylated and arrestin-bound receptor, along with a previously membrane-bound G protein-coupled receptor phosphatase (GRP), is engulfed into the maturing endosome. The acidic pH of the endosome (pH ∼ 5–6) then causes a change in the receptor conformation that enables the GRP to bind and dephosphorylate the receptor. Following subsequent dissociation of the ligand and the arrestin, the re-sensitized receptor either travels back to the plasma membrane or is degraded in the lysosomes [437,453].

Ligand-induced internalization and recycling of GLP-1R has been demonstrated in Chinese hamster lung fibroblasts [453], in rat insulinoma cells [429,453,454], and in rat pancreatic BRIN BD11 cells [452]. *In vitro* studies using 125I-labeled GLP-1 (7-36amide) in rat insulinoma cells indicate that internalization of the ligand-GLP-1R complex is saturable and time- and temperature-dependent [454]. At the plasma membrane, GLP-1R recruitment of arrestin is observed as early as 1 min after stimulation with exendin-4 [452]. Surface re-expression of GLP-1R after ligand-induced GLP-1R endocytosis appears with a half-time of 15 min with no GLP-1R endocytosis upon treatment of cells with exendin (9–39) [453]. Notably, in the endosomes, the receptor–ligand complex co-localizes with adenylate cyclase. Pharmacological inhibition of GLP-1R endocytosis by dynasor attenuates cAMP formation in BRIN BD11 pancreatic β-cells and lowers PKA substrate phosphorylation, resulting in a lesser magnitude of glucose-stimulated insulin secretion [450]. These data are consistent with reports that the internalized GLP-1R is to a certain extent still capable of stimulating insulin secretion from pancreatic β-cells [451]. Continued cAMP generation after GLP-1R sequestration (internalization) is accompanied by joint translocation of the activated Gαs subunit along with GLP-1R into the endosome [452]. Interestingly, generation of cAMP by the internalized GLP-1R persists after recruitment of arrestin, which indicates that arrestin recruitment is not essential for GLP-1R desensitization [452]. In contrast to these findings, siRNA mediated knockdown of β-arrestin in INS-1 cells attenuates GLP-1-induced phosphorylation of CREB, ERK1/2, and IRS-2 and diminishes the insulinotropic effect of GLP-1 in conditions of low (2.5 mM) and high glucose (25 mM) [429]. These studies suggest that recruitment of β-arrestin following ligand-induced activation of GLP-1R is important for the insulinotropic effect of GLP-1. However, knockdown of β-arrestin has minimal effect on GLP-1R internalization or desensitization [429]. In one recent report, exendin-4 analogs were modified on their N-terminal end to modulate GLP-1R trafficking and/or signaling [455]. The insulinotropic efficacy of these biased-signaling molecules was inversely correlated to the receptor internalization; i.e. insulin release was greatest using molecules that retain GLP-1R at the cell surface [455].

In summary, accumulating evidence indicates that ligand-induced GLP-1R signaling is partially preserved after endocytosis of the receptor. Consistent with this, continuous cAMP formation has been demonstrated upon internalization of the receptors for parathyroid hormone [456], thyroid-stimulating hormone [457], and sphingosine 1-phosphate [458]. The exact mechanisms underlying continuation of receptor signaling after sequestration of the ligand–receptor complex seemingly involve preserved association of the receptor with the Gαs subunit. The residence time of the ligand interaction with the receptor is commonly hypothesized to affect the duration an internalized receptor can continue to signal [459].

7.5. Studies using GLP-1R-deficient animals

Mice globally deficient for GLP-1R are viable and have no overt metabolic phenotype when fed a standard chow diet ad libitum [460–462]. However, these mice are mildly hyperglycemic under conditions of fasting and display impaired glucose clearance during oral and intraperitoneal glucose tolerance tests (OGTT and ipGTT), with decreased glucose-stimulated insulin secretion [460,461] and normal pre- and postprandial levels of glucagon [461]. Although the incretin effect is diminished in GLP-1R KO mice, these mice have normal body weight and food intake on regular chow diets [460–462]. When chronically fed a high-fat diet (HFD), GLP-1R KO mice are paradoxically leaner than their wild-type controls yet are more glucose intolerant [462]. The molecular mechanism underlying the diminished insulinotropic response of the GLP-1R KO mice to orally administered glucose likely reflects loss of GLP-1R-dependent augmentation of insulin secretion. While GLP-1 enhances proinsulin expression [82,372,373,463,464], there are no differences in pancreatic insulin expression in GLP-1R KO mice [461]. Consistent with the hypothesis that GLP-1R is the only endogenous receptor for GLP-1, either peripherally [465–467] or centrally [460] applied GLP-1R agonists fail to affect glycemic control, body weight, and food intake in mice globally deficient for GLP-1R. In summary, the most obvious metabolic phenotype of the global germline GLP-1R KO mice is diminished insulin secretion in response to oral glucose, and a paradoxical protection from diet-induced obesity despite impaired glycemic control.

8. MOLECULAR MECHANISMS UNDERLYING GLP-1-INDUCED INSULIN SECRETION

Induction of glucose-dependent insulin secretion by GLP-1 has been demonstrated *in vivo* in numerous species including rodents [468–471] and humans [83] as well as in the isolated perfused pancreas of rats [81,472–474], dogs [473], pigs [80,475], and humans [77], and in isolated islets [476] and cultured pancreatic cell lines [82,370–373,472,477–479]. GLP-1 (7–37) is roughly 100-fold more potent than glucagon to stimulate the secretion of insulin [480]. Nevertheless, intra-islet glucagon levels are much higher than circulating levels, and studies in mice highlight the importance of glucagon, acting through the GLP-1 receptor in the control of glucose-stimulated insulin secretion [173,365]. The key importance of GLP-1 and GIP in stimulating insulin secretion under conditions of hyperglycemia is the finding that oral glucose-induced insulin secretion is more impaired in mice that lack both incretin hormone receptors [481–483].

8.1. Acute insulinotropic effects of GLP-1

In β-cells, binding of GLP-1 to its receptor leads to activation of adenylate cyclase (AC) and subsequently to an increase in cAMP (Figure 5) [82]. Overexpression of GLP-1R in rat insulinoma RIN1046-38 cells results in elevated levels of basal cAMP [484]. While both GLP-1 (1–37) and GLP-1 (7–37) stimulate insulin secretion, GLP-1 (7–37) is efficacious at a lower dose. At a dose of 5 μM, both GLP-1 (1–37) and GLP-1 (7–37) increase cAMP levels in RIN1046-38 cells but at a dose of 5 nM, only GLP-1 (7–37) enhances cAMP levels [82]. Consistent with the increase in cAMP, GLP-1 stimulation of insulin mRNA is substantially greater upon treatment of RIN1046-38 cells with GLP-1 (7–37) relative to treatment with GLP-1 (1–37) [82]. Further supporting the greater insulinotropic potency of GLP-1 (7–37) relative to GLP-1 (1–37), GLP-1 (7–37) stimulates insulin release in the
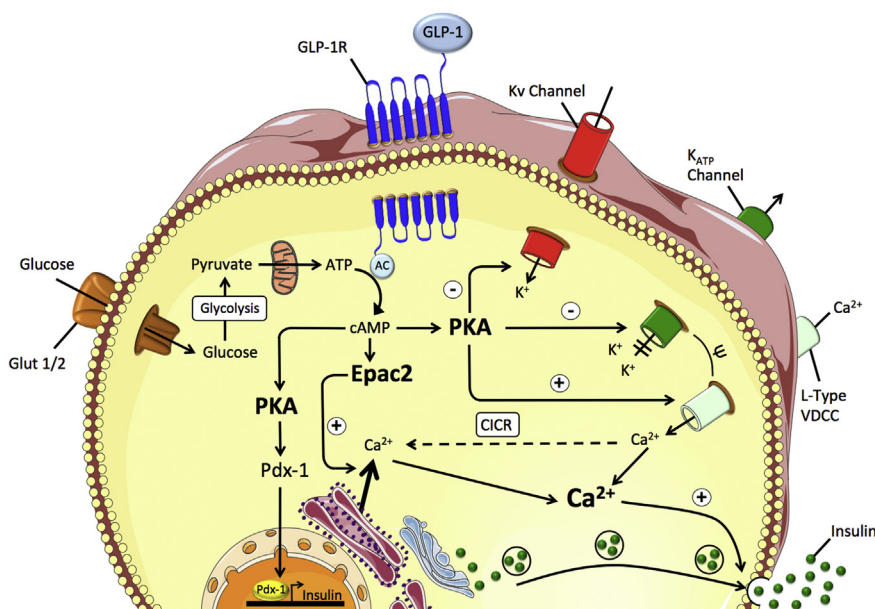
Figure 5: Schematic on GLP-1 mediated insulin secretion in the β-cell. GLUT1/2: glucose transporter 1/2; AC: adenylate cyclase; PKA: protein kinase A; Epac2: exchange protein activated by cAMP; Pdx-1: pancreatic and duodenal homeobox 1; CICR: calcium-induced calcium release. For further explanations, please see text.

perfused rat pancreas at a concentration of 50 pM whereas treatment with GLP-1 (1—37), even at a dose of 0.5 μM, has no effect on insulin secretion [81]. Underlining the role of cAMP in the insulinotropic effect of GLP-1, enhanced cAMP hydrolysis through overexpression of the cyclic nucleotide phosphodiesterase 3B (PDE3B) diminishes GLP-1-induced insulin secretion [485]. Insulin and IFG-1 increase levels of PDE3B, and treatment of hamster clonal β-cells (HIT-T15) with IGF-1 deteriorates the insulinotropic effect of GLP-1 [486]. Transgenic mice overexpressing PDE3B under control of the rat insulin 2 promoter are glucose intolerant and have impaired insulin secretion following an intravenous glucose challenge [487]. In summary, there is substantial evidence indicating that cAMP is an important second messenger driving the acute insulinotropic effect of GLP-1.

The GLP-1-induced rise in cAMP leads to activation of PKA and enhanced signaling via exchange proteins directly activated by cAMP (Epac) (Figure 5) [488—490]. cAMP activates PKA, which phosphorylates the β_2 subunit of the L-type VDC channels and potentially the Kir6.2 and SUR1 subunits of the K_{ATP} channels [491]. This consequently increases the sensitization of the K_{ATP} channels to ATP, leading to closure of the K_{ATP} channels, depolarization of the cell membrane, and opening of VDC channels (Figure 5). The subsequent Ca^{2+} influx promotes exocytosis of the insulin granules and acute secretion of insulin into the circulation. At the same time, the GLP-1-activated PKA inhibits voltage-gated K^+ (K_V)-channels, preventing membrane repolarization and boosting Ca^{2+} influx via prolonged opening of the VDC channels (Figure 5) [492]. Inhibition of PKA in isolated islets attenuates GLP-1-induced insulin secretion [490].

The acute insulinotropic effect of GLP-1 does not fully depend on PKA signaling [493]. Up to 50% of the GLP-1-induced insulin release can be mediated through signaling via Epac [489,494,495]. Similar to PKA, members of the Epac family contain an evolutionarily conserved cAMP binding domain which enables the members of this family (Epac1 and Epac2) to regulate diverse biological functions in a cAMP-dependent manner [496]. Both Epac1 and Epac2 are expressed in the pancreas [495,497]. In β-cells, the Epac proteins stimulate Ca^{2+} release from the endoplasmic reticulum (ER), increasing insulin secretion by increasing the intracellular pool of Ca^{2+} [488]. cAMP activates Epac2 through direct binding. In high glucose conditions where there is an enhanced Ca^{2+} influx into the β-cells via the VDC channels, Epac2 opens RYR Ca^{2+} channels in the ER, further increasing intracellular Ca^{2+} levels, and potentiates insulin exocytosis (Figure 5) [489]. The GLP-1/Epac2 mediated opening of the RYR Ca^{2+} channels in the ER depends on the simultaneous Ca^{2+} influx via the VDC channels. This process, generally referred to as calcium-induced calcium release (CICR) (Figure 5) [489,498—500], ensures that the insulinotropic action of GLP-1 is highly dependent on the ambient glucose concentration. Accordingly, in the isolated perfused rat pancreas, GLP-1 fails to stimulate insulin release at glucose concentrations <2.8 mM, but amplifies insulin release at glucose concentrations >6.6 mM [82,480]. Consistent with this, clinical studies demonstrate that GLP-1R agonism improves glycemic control in patients with type 2 diabetes but with little risk of hypoglycemia when GLP-1 is used without accompanying sulfonylurea therapy [501—507].

Beyond its ability to increase the intracellular pool of Ca^{2+}, GLP-1 also affects insulin secretion via cAMP-mediated regulation of exocytosis (Figure 6). In isolated murine β-cells, GLP-1 stimulates exocytosis of the insulin granules and this can be blocked by co-treatment of GLP-1 with the adenylate cyclase inhibitor MDL 12330A [508]. As reviewed previously [508], the insulin granules are organized in distinct pools that vary in their release competence. Most granules (>99%) belong to the immature "reserve" pool that must undergo a series of maturation processes to acquire release-competence. Only 0.5—1% of the granules belong to the readily releasable pool (RRP), from which only a subset, the immediately releasable pool (IRP), is located in the vicinity of the Ca^{2+} channels and is released with minimal time delay upon opening of the Ca^{2+} channels [508]. cAMP enhances insulin exocytosis by promoting maturation of the granules and thus by increasing the pool of release-competent (RRP/IRP) granules [508—510].

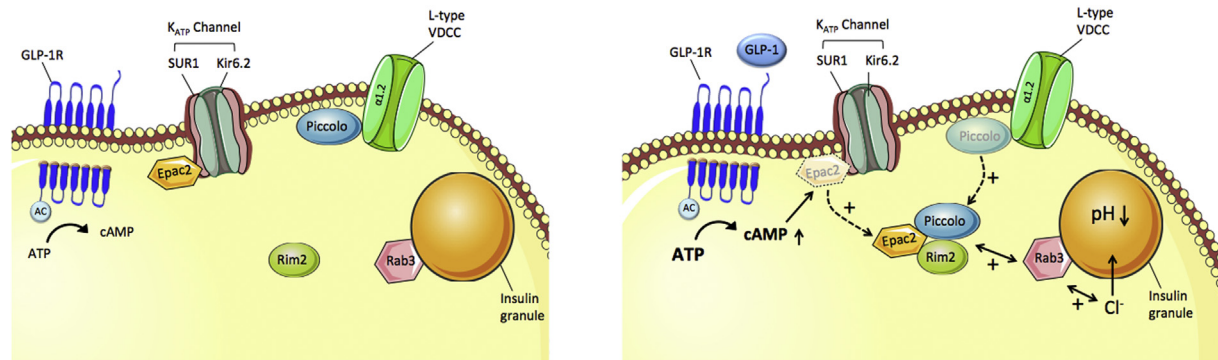www.molecularmetabolism.com

Review

Figure 6: Schematic on GLP-1 mediated exocytosis of the insulin granules in the β-cell. For further explanations, please see text.

cAMP regulates exocytosis via PKA-dependent and -independent mechanisms. GLP-1-stimulation of exocytosis is partially inhibited when isolated mouse β-cells are treated with the PKA inhibitor H89 but is fully inhibited upon co-treatment with H89 with an antiserum against Epac2 (a.k.a. cAMP-GEFII) [508]. Epac2 binds to the nucleotide binding domain-1 of the SUR1 subunit of the K_{ATP} channel and this interaction is aborted when levels of cAMP increase (Figure 6) [511]. Apart from binding SUR1, Epac2 also binds to a series of molecules that potentially participate in transport, priming and fusion of the insulin vesicles, including Rab3, Rim2, and the Ca^{2+} sensor Piccolo [493,512,513]. Epac2 interaction with Rim2 and Piccolo is crucial for cAMP-induced Ca^{2+}-dependent insulin secretion and is not blocked by inhibition of PKA [493,512,513]. A hypothetical model for the regulation of exocytosis by Epac2 is that the GLP-1-induced rise in cAMP leads to a conformational change of Epac2, which then dissociates from SUR1 and stimulates dissociation of Rim2 from the $α1.2$ subunit of the L-type Ca^{2+} channel (Figure 6). The dissociated Epac2 then dimerizes with Rim2 and Piccolo in a Ca^{2+}-dependent manner followed by induction of exocytosis of the insulin granules through binding of the Epac2/Rim2/Piccolo complex to Rab3 which is located at the membrane of the insulin granules (Figure 6) [508,511]. Another PKA-independent mechanism of GLP-1 stimulation of insulin secretion includes PKC-dependent membrane depolarization via activation of transient receptor potential cation channel subfamily M member 4 (Trpm4) and Trpm5 [514]. In murine and human islets, GLP-1, via phospholipase C (PLC), leads to an activation of PKC, followed by membrane depolarization and stimulation of insulin secretion. Membrane depolarization by GLP-1 was mimicked by the PKC activator phorbol myristate acetate (PMA) and was not affected by treatment with either the PKA inhibitor myr-PKI, the K_{ATP} channel blocker tolbutamide, or the L-type Ca^{2+} channel inhibitor isradipine [514]. The PKC-dependent effect of GLP-1 on membrane depolarization was mediated by activation of Trpm4 and Trpm5, and GLP-1 failed to increase electrical activity and insulin secretion in islets isolated from Trpm4 and Trpm5 KO mice [514]. Another recent report suggests that incretin-stimulation of insulin secretion requires incorporation of glutamate in the insulin granules. In murine pancreatic Min6-K8 cells, prevention of cytosolic glutamate production by KO of a key component of the malate-aspartate shuttle or triple KO of the vesicular glutamate transporters Slc17a6, Slc17a7, and Slc17a8 impaired GLP-1-induced insulin secretion [515].

GLP-1 also lowers blood glucose in patients with type 1 diabetes, e.g. via its ability to slow down gastric emptying, indicating that not all of GLP-1s glycemic effects derive from its action on the β-cell or insulin

action [516,517]. Post-prandial levels of portal blood glucose are elevated and insulin levels decreased in rats with knockdown of GLP-1R in vagal afferent neurons, suggesting that GLP-1 also regulates blood glucose via activation of vagal afferent GLP-1 receptors [518]. These data are consistent with a recent report showing that GLP-1 stimulation of insulin secretion requires GLP-1R and neuronal nitric oxide synthase in the enteric nervous system [519]. Moreover, mice with targeted inactivation of GLP-1R in autonomic neurons targeted by Phox2b, including the nodose ganglion, exhibit impaired glucose homeostasis, accelerated gastric emptying, and increased levels of GLP-1, glucagon, and insulin, supporting the importance of vagal afferent GLP-1Rs for metabolic homeostasis [520].

8.2. Effects of GLP-1 on (Pro-)insulin synthesis

In addition to the ability of GLP-1 to stimulate insulin secretion via signaling through PKA and Epac2, GLP-1R agonism also increases glucose metabolism by promoting insulin synthesis (Figure 5). GLP-1 stimulation of insulin gene expression was first reported by Daniel Drucker in rat insulinoma RIN1046-38 cells [82] and was subsequently confirmed in several independent studies [372,373,463,464]. In RIN 1046-38 cells, the GLP-1-induced rise in insulin mRNA is accompanied by an increased expression of GLUT1 and hexokinase1 [464]. Treatment with the transcription inhibitor actinomycin D blocks GLP-1 stimulation of GLUT1 and hexokinase1 expression in these cells without affecting GLP-1 induction of insulin expression [464]. These data led to the hypothesis that the increased insulin mRNA levels occurring upon GLP-1 exposure might be due to stabilization of the mRNA, whereas the elevated expression of GLUT1 and hexokinase are due to direct transcriptional stimulation by GLP-1 [464]. Nevertheless, GLP-1 also activates proinsulin gene transcription in islet insulinoma cells [372,521]. Notably, induction of proinsulin expression by either GLP-1 or forskolin can be blocked by galanin, suggesting that GLP-1 stimulation of insulin expression is cAMP dependent [373].

GLP-1-induced stimulation of insulin synthesis is initiated by Pdx1, a transcription factor implicated in development of the pancreas [490] and in MODY type-4 diabetes [522,523]. GLP-1 induced activation of PKA increases Pdx1 expression and translocation of Pdx1 to the nucleus, where Pdx1 binds to the insulin promoter to initiate insulin expression and synthesis (Figure 5) [490,524]. As demonstrated in RIN1046-38 cells, PKA activation of Pdx1, but not glucose-induced Pdx1 nuclear translocation, can be blocked either by PKA inhibition or by using a cAMP antagonist, implying that GLP-1 regulates the PKA/Pdx1 axis in a cAMP-dependent manner [490]. Accordingly, GLP-1 effects on Pdx1 can be mimicked by treatment of RIN1046-38 cells

with either forskolin or the cAMP analog 8-Bromo-cAMP [490]. In summary, GLP-1 stimulates both the synthesis and secretion of insulin via multiple cAMP-dependent pathways.

9. GLP-1 EFFECTS ON β-CELL PROLIFERATION AND APOPTOSIS

The prevalence of type 2 diabetes correlates with excess body weight and also increases with age [525]. The progression to type-2 diabetes is invariably associated with a decline in functional β-cell mass [526—530]. Decreased β-cell proliferative capacity is also age-dependent in rodents and humans [526,531—537]. The replication rate of human β-cells is greatest in young childhood and puberty but declines with increasing age [526,531,533,536]. Collectively, these observations suggest that age-related changes in β-cell neogenesis and replication might be causally linked to the development of type 2 diabetes [525,532,538]. A role for β-cell dedifferentiation has also been proposed [539].

Agonists of the GLP-1 receptor improve glycemic control via both their acute insulinotropic action and, under certain circumstances, also by chronic action to preserve β-cell mass through stimulation of β-cell proliferation and inhibition of apoptosis (Figure 7) [540—545]. While inconsistent results have been reported, the treatment duration, age of the animal, and species under investigation as well as diet-composition might affect the ability of GLP-1R agonism to

improve β-cell replication [540]. GLP-1R regulation of β-cell proliferation and apoptosis seems to engage mechanisms that include signaling via Pdx1. Exendin-4 stimulates β-cell proliferation and inhibits β-cell apoptosis in wildtype mice but not in mice with β-cell specific inactivation of Pdx1 [542]. GLP-1R agonism also promotes β-cell growth and survival by stimulating the expression of the insulin receptor substrate 2 (Irs2) via mechanisms that include activation of CREB [546]. Irs2 is a substrate of the IGF1 and insulin receptor tyrosine kinases that promote growth, function and survival of the β-cells [547]. Consistent with this role of Irs2, increased expression of Irs2 in β-cells improves insulin secretion in obese mice and protects against STZ-induced β-cell destruction (as reviewed in [547]). Mice deficient for Irs2 [544], or transgenic mice deficient in CREB activity [546], are hyperglycemic due to severe β-cell destruction and enhanced β-cell apoptosis. Exendin-4 improves Irs2 function by enhancing CREB phosphorylation *in vitro* and *in vivo* [546]. Chronic administration of exendin-4 is unable to prevent β-cell loss in mice that are lacking Irs2 [544], demonstrating the requirement of Irs2 to GLP-1-mediated β-cell plasticity. Stimulation of β-cell proliferation secondary to stimulating GLP-1R is usually only observed in young animals (during periods characterized by preserved proliferative capacity), and not in older rodents [548]. Solid clinical proof of slowed diabetes progression or enhanced β-cell mass has not been provided in type-2 diabetic patients treated with any GLP-1 receptor agonist [549].
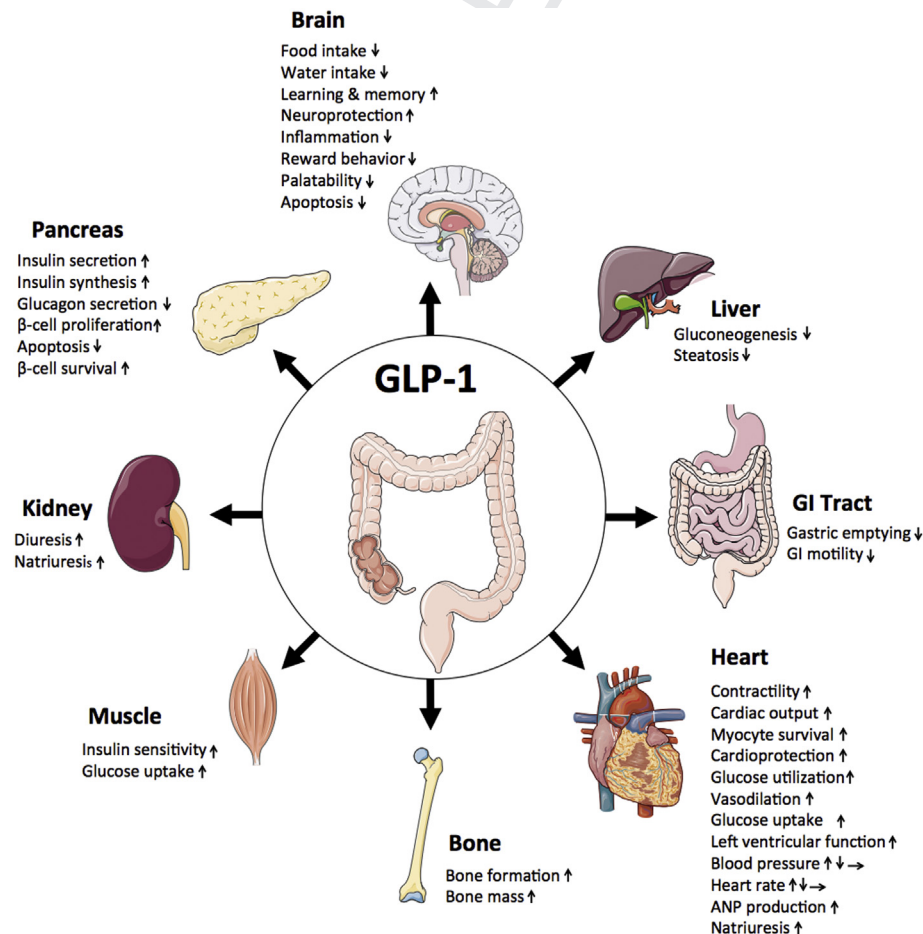

Figure 7: Schematic on the metabolic effects of GLP-1. The shown effects include direct and indirect GLP-1 effects on metabolism. For further explanations, please see text.

Review

The anti-apoptotic effect of GLP-1R agonism has been demonstrated *in vivo* in mice [543,550,551] and rats [552,553] as well as in several rodent [550,551] and human [554,555] cell lines, purified rat β-cells [543], and humans [556]. STZ-induced β-cell apoptosis is reduced upon administration of exendin-4 [543] or GLP-1 (7—36 amide) [551] and treatment with exendin-4 diminishes hyperglycemia induced by STZ [543]. STZ-induced β-cell apoptosis is accelerated in mice deficient for the GLP-1 receptor [543]. In isolated rat β-cells, treatment with exendin-4 reduces the apoptotic effect induced by treatment with pro-apoptotic cytokines, such as IL1b, TNFa, and interferon gamma [543]. In mouse pancreatic βTC-6 cells, liraglutide enhances β-cell survival via stimulation of anti-apoptotic signaling mechanisms that include stimulation of PI3 kinase-dependent phosphorylation of AKT, leading to inactivation of the pro-apoptotic protein BAD and silencing of FoxO1 [550].

An innovative approach to assess human β-cell replication *in vivo* was recently established by the group of Alvin Powers [557]. In this model, human β-cells were grafted under the renal capsule of lean diabetic immunodeficient NOD scid gamma mice. Using this model, exendin-4 was found to enhance β-cell proliferation in juvenile but not adult human islets [557]. Notably, the age-dependent decline in the exendin-4 induced β-cell proliferation was not related to changes of GLP-1R expression, as also indicated by preservation of exendin-4-induced insulin secretion in the adult islets [557]. However, in juvenile but not adult human islets, exendin-4 stimulated calcineurin/NFAT signaling and enhanced expression of proliferation-promoting factors such as NFATC1, FOXM1, and CCNA1 [557]. [557]. Collectively, these data suggest that the sensitivity of β-cell proliferative mechanisms to respond to GLP-1R agonism declines with increasing age [557].

10. IMPAIRED RESPONSIVENESS OF GLP-1 TO STIMULATE INSULIN SECRETION

GLP-1-stimulated insulin secretion has an estimated heritability of 0.53, as demonstrated in twin studies [558]. The first evidence for an influence of genetic background on GLP-1-induced insulin secretion was found for variation in the transcription factor 7-like 2 (*TCF7L2*) [559]. Infusion of GLP-1 during a classical hyperglycemic clamp in non-diabetic individuals results in a marked reduction of insulin secretion in carriers of the *TCF7L2* risk allele of SNP rs7903146. Diminished insulin secretion in response to treatment with GLP-1 has been replicated in other studies [560—562].

In addition to TCF7L2, several other genetic loci have been identified to be associated with reduced GLP-1-induced insulin secretion, including the loci for *GLP-1R* [563], wolfram syndrome 1 (*WFS1)* [564] and chymotrypsinogen B1/2 (*CTRB1/2*) [565]. The molecular mechanisms leading to diminished GLP-1 responsiveness associated with genetic variation in *TCF7L2* most likely includes alterations of the WNT signaling pathway and associated β-cell proliferation and insulin gene expression [566]. Interestingly, genetic variation in a transcriptional regulator of the insulin gene nuclear receptor subfamily 4 group A member 3 (Nor-1) is able to rescue *TCF7L2*-mediated GLP-1 resistance [567]. Impairment of proinsulin conversion may be another mechanism for *TCF7L2* associated diminished GLP-1 efficacy [568]. Another plausible mechanistic explanation for a diminished "incretin effect" is the *TCF7L2*-dependent repression of GLP-1R and GIPR expression on the β-cells [569]. The association between *WFS1* variants and impaired incretin action may result from alterations of endoplasmic reticulum homeostasis and, consequently, β-cell dysfunction [566]. Recently, using an untargeted integrative genomics approach, a set of genes that contained variants associated with GLP-

1-stimulated insulin secretion were described, which at the same time have the potential to physically interact in the β-cell and are enriched for pathways that are important for insulin secretion [570]. Finally, the GLP-1 effects on insulin secretion are dependent on the metabolic state of the individual. Incretin action may also be reduced during hyperglycemia and in some individuals with diabetes, prediabetes and insulin resistance [571].

In summary, a reduced incretin effect on insulin secretion is associated with both genetic and metabolic alterations. The presence of both hyperglycemia and genetically determined GLP-1 resistance can potentiate the impairment of GLP1-induced insulin secretion [572].

11. GLP-1 EFFECTS ON GLUCAGON SECRETION

GLP-1 also decreases blood glucose by suppressing glucagon secretion (Figure 7) [475,573,574]. GLP-1 inhibition of glucagon secretion has been demonstrated *in vivo* in numerous species including mice [575], dogs [576], and humans [516,517,573,577—581], as well as in the isolated perfused pancreas of rats [473,474,582], dogs [473], and pigs [475] and in isolated intact murine islets [583]. Clamp studies in patients with type 2 diabetes indicate that GLP-1 inhibition of glucagon secretion is equally important for lowering blood glucose as GLP-1 stimulation of insulin release [578].

The mechanisms underlying GLP-1 suppression of glucagon secretion are complex. In the isolated perfused pig pancreas, GLP-1 dose-dependently stimulates the secretion of somatostatin [475] which is a potent suppressor of glucagon secretion [584]. Somatostatin inhibits glucagon secretion via paracrine mechanisms and, when blocked, stimulates glucagon release in isolated rat islets [585,586]. In the isolated perfused rat pancreas, co-infusion of GLP-1 with a specific somatostatin receptor 2 (SSTR2) antagonist (PRL-2903) abolishes the GLP-1-induced suppression of glucagon secretion [582]. While these data point to a prominent role of somatostatin in mediating GLP-1 suppression of glucagon secretion, treatment of isolated murine islets with the SSTR2 antagonist CYN154806 does not fully inhibit GLP-1's ability to suppress glucagon secretion [583]. Consistent with the conclusion that GLP-1 suppression of glucagon secretion does not fully depend on somatostatin, GLP-1 effects on glucagon secretion can be mimicked by forskolin-induced changes in cAMP. Accordingly, treatment of isolated murine islets with low concentrations of forskolin (1—10 nM) suppresses glucagon secretion by up to 60%, while high concentrations of forskolin (0.1—10 μM) stimulate glucagon release [583]. Notably, the PKA inhibitor 8-Br-Rp-cAMPS attenuates the inhibitory effect of GLP-1 on glucagon secretion, suggesting that GLP-1 suppression of glucagon secretion is PKA-dependent [583]. In intact murine islets, blockade of N-type Ca^{2+} channels with ω-conotoxin, but not L-type Ca^{2+} channels using nifedipine, abolishes low glucose (1 mM) stimulation of glucagon secretion and blunts the GLP-1-mediated inhibitor effects. In summary, these data indicate that GLP-1 may inhibit α-cell glucagon secretion via PKA-dependent modulation of N-type Ca^{2+} channel activity in addition to its paracrine action via somatostatin [583].

GLP-1 may also inhibit glucagon secretion indirectly via its insulinotropic effect on the β-cells. Accordingly, as described in recent review articles [4,574], GLP-1 stimulates both the δ-cells to secrete somatostatin and the β-cells to secrete insulin, amylin, zinc, and GABA, all of which suppress the release of glucagon. In α-cell-derived IN-R1-G9 cells, insulin inhibits glucagon release via activation of phosphatidylinositol 3-kinase (PI3K). Inhibition of PI3K by wortmannin offsets the ability of insulin to suppress glucagon secretion [587]. In α-cells, insulin further enhances translocation of GABA-A receptors [588], and

GABA released from β-cells enhances glucose inhibition of glucagon secretion [589]. Insulin is co-crystalized with Zn^{2+} in the secretory granules of the β-cells [590,591] and Zn^{2+} is co-secreted with insulin under conditions of hyperglycemia [592,593]. Zn^{2+} acts in a paracrine manner on the α-cells to inhibit the secretion of glucagon [593—595]. Interestingly, disruption of the intrapancreatic infusion of Zn^{2+} but not of Zn^{2+} free insulin accelerates glucagon secretion in rats made diabetic by STZ, indicating that Zn^{2+} but not insulin is the main stimulus underlying the Zn^{2+}-insulin inhibition of glucagon secretion [595]. Consistent with this, glucagon secretion is inhibited upon treatment of α-TC cells with Zn^{2+} [596]. As demonstrated in isolated rat α-cells, intact islets and the perfused rat pancreas, Zn^{2+} inhibits pyruvate-induced glucagon secretion via opening of K_{ATP} channels and inhibition of α-cell electrical activity [593]. In summary, there is credible evidence indicating that Zn^{2+} that is co-secreted with insulin from the β-cells plays a prominent role in inhibiting release of glucagon [593—595]. Amylin, which is also co-secreted with insulin, also affects glucagon release from the α-cells. Amylin dose-dependently suppresses arginine-mediated glucagon secretion in rats [597] whereas pharmacological inhibition of amylin signaling enhances glucagon secretion [598]. Pramlintide, a synthetic amylin receptor agonist, improves glycemic control in diabetic patients via inhibition of postprandial glucagon secretion and inhibition of gastric emptying [599,600]. Interestingly, amylin does not affect glucagon secretion in isolated islets [601] nor in the perfused rat pancreas [602,603], suggesting that amylin regulation of glucagon is not cell autonomous. Notably, while GLP-1 can potentially affect glucagon secretion via its stimulatory effect on the β-cells in normal physiological conditions, GLP-1 also inhibits glucagon release in type 1 diabetes, thus demonstrating that GLP-1 inhibition of glucagon secretion does not fully depend on the β-cell secretome [516].

In summary, GLP-1 potently inhibits glucagon secretion. This occurs in part by a paracrine stimulatory effect on the islets to secrete somatostatin, and perhaps insulin, Zn^{2+}, GABA, and amylin, at elevated glucose concentrations. GLP-1 likely suppresses glucagon secretion via endocrine rather than direct mechanisms. Most studies report the absence [397—399] or very restricted expression of the GLP-1 receptor in a small subset (10%) of α-cells [395], and treatment of isolated rat α-cells with GLP-1 enhances rather than inhibits the release of glucagon [604—606]. GLP-1 may nevertheless also directly inhibit glucagon secretion, because α-cell-specific GLP-1R KO mice have elevated non-fasting glucagon levels relative to wildtype controls and female α-cell-specific GLP-1R KO mice are mildly glucose intolerant with increased secretion of glucagon upon peripheral administration of glucose [607]. Further supporting a direct GLP-1 inhibition of glucagon secretion is the fact that such secretion is not prevented in isolated human islets treated with the insulin receptor antagonist S961 or the somatostatin receptor 2 antagonist CYN154806 [608].

12. CARDIOVASCULAR EFFECTS OF GLP-1

The presence of GLP-1R mRNA in the heart was first demonstrated in rats [380] and humans [381,609]. Expression was subsequently confirmed at the protein level [201,610] and histologically using mice that express GFP under control of the GLP1-R promoter. In these mice scattered GLP-1R expression was detected in cardiomyocytes of the atrium but not in the ventricles, and widespread expression was detected in smooth muscle cells of coronary vessels [395]. As discussed in a comprehensive review article [611], the anatomical location of GLP-1R within the cardiovascular system has species-related differences. In mice, GLP-1R immunoreactivity using multiple

different antisera is found in the cardiomyocytes, endocardium, microvascular endothelium, and the smooth muscle cells in the coronary arteries [201]. In humans, the presence of GLP-1R has primarily been demonstrated in the endothelium, the coronary arteries and the smooth muscle cells [381,612]. Nevertheless, there is little concordance between the available localization data from immunohistochemistry vs. detection of GLP-1R mRNA in situ hybridization. In view of the many specificity problems associated with antibodies against the GLP-1 receptor, some of the incongruent findings may also result from lack of antisera specificity. Using well-characterized antibodies, expression was noted in the non-human primate and human sinoatrial node [392], which would be consistent with the effect of GLP-1 on heart rate. Surprisingly, in one recent study, robust expression of the GLP-1 receptor mRNA was also reported in human cardiac ventricles, but the exact localization of the GLP-1R protein could not be determined [613].

Although best known for its insulinotropic and weight lowering action, GLP-1R agonism also confers a series of beneficial effects on the cardiovascular system in rodents. These include an increase of cardiomyocyte survival via inhibition of apoptosis [614,615], amelioration of endothelial dysfunction [612,616], improvement of regional, and global cardiac output following injury and heart failure [614,617—619] (Figure 7). Reduction of blood pressure is also reported in hypertensive humans [620]. Prolonged treatment with liraglutide further improves cardiovascular outcome in patients with T2DM [621]. Of note, GLP-1 improvement of cardiovascular performance is, at least in part, independent of its ability to decrease body weight and to improve lipid metabolism, and GLP-1 may promote its cardiac effects via direct action at GLP-1R as well as indirect mechanisms independent of cardiac GLP-1R action [201,611,622].

12.1. Effects of GLP-1 on cardiac performance after cardiac injury

Endothelial dysfunction is a common co-morbidity associated with insulin resistance and T2DM. Treatment of type-2 diabetic patients that present coronary artery disease with GLP-1 improves endothelial function without affecting insulin resistance [612]. Similar findings have been reported for GLP-1 treatment of type-1 diabetes [623] but GLP-1 improvement of endothelial function seems to be related to native GLP-1 and is not observed when GLP-1R agonists are compared head-to-head with equally glucose lowering medications.

GLP-1 improvement of endothelial function, assessed by flow-mediated vasodilation, was also demonstrated during a hyperglycemic clamp in patients with T2DM and normoglycemic controls [616]. In rats, GLP-1 (7-36amide) and, perhaps paradoxically, the GLP-1R antagonist exendin (9—39), dose-dependently cause relaxation of the aorta, potentially via elevation of cAMP formation and activation of K_{ATP} channels [624].

In dogs with acute myocardial infarction, infusion of GLP-1 improves cardiac performance by increasing myocardial glucose uptake and by enhancing left ventricular function [617]. In the same animal model, infusion of GLP-1 limited myocardial stunning (ventricular dysfunction without myocardial necrosis) following reperfusion [618]. These data are in agreement with non-controlled clinical studies reporting that 72 h of native GLP-1 infusion improved regional and global left ventricular performance in patients with acute myocardial infarction and severe systolic dysfunction [619]. In mice, 7-day treatment with liraglutide prior to induction of myocardial infarction increases survival and reduces cardiac injury and infarction size, while improving cardiac output [614]. In the isolated perfused rat heart, acute GLP-1R agonism protects from ischemia/reperfusion injury [625,626], an effect that can be blocked by pretreatment with either exendin (9—39), the cAMP

Review

inhibitor Rp-cAMP, the PI3 Kinase inhibitor LY294002 or the MAP Kinase inhibitor UO126 [625]. The cardioprotective effect of GLP-1R agonism seems to be, at least in part, independent of GLP-1-induced weight loss, dependent on the canonical GLP-1R, and accompanied by enhanced expression of cardioprotective genes in the heart, including Akt, GSK3β, PPARΔ, NRF-2, and HO-1 [614]. Treatment of primary neonatal mouse cardiomyocytes with liraglutide increases cAMP formation and reduces apoptosis, as indicated by inhibition of TNFa induction of caspase 3 activity [614]. GLP-1R agonism has direct cell autonomous anti-apoptotic effects in the rodent heart, and GLP-1 reduces hypoxia-induced apoptosis in isolated neonatal rat cardiomyocytes, and inhibition of PI3K using LY29400 or MAPK using UO126 blocks the anti-apoptotic effect of GLP-1 [615]. GLP-1 has different effects on left ventricular function and cardiac contractility under healthy vs. pathological conditions. GLP-1-induced improvement of left ventricular function has been reported in dogs with cardiomyopathy [617], in patients with acute myocardial infarction [619], and in the isolated mouse heart [201]. At least part of the improvement in cardiac output may be driven by an increase in heart rate. Other studies, however, report failure of GLP-1 to directly accelerate contraction in isolated adult rat cardiomyocytes, notably despite GLP-1 stimulation of cAMP accumulation [627]. Further, treatment of the healthy isolated rat heart with GLP-1 has been reported to reduce left ventricular contractility despite GLP-1 stimulation of myocardial glucose uptake [628]. However, as demonstrated in the same study, GLP-1 enhances recovery of the heart after ischemia and improves left ventricular function and myocardial glucose uptake [628]. GLP-1R KO mice have increased baseline left ventricular developed pressure (LVDP) during perfusion [201]. Mice deficient for GLP-1R have decreased resting HR and an increased thickness of the left ventricular wall relative to wildtype controls [201,629], as well as reduced cardiac contractility when challenged with insulin or epinephrine [629]. Collectively, these data indicate that GLP-1 improves cardiac performance and output in the post-ischemic heart but otherwise may decrease cardiac output under non-pathological conditions. Although mice with cardiomyocyte-specific deletion of GLP-1R have lower basal heart rate (HR), they fully retain the cardioprotective potential of GLP-1R agonism following left arterial descending (LAD) coronary artery occlusion [630]. These data suggest that cardiomyocyte GLP-1R signaling is required for regulation of HR but is unrelated to the cardioprotective effects of GLP-1R agonism following cardiac injury.

12.2. Cardiometabolic effects of GLP-1(9-36amide)
Accumulating evidence indicates that some cardiac effects of GLP-1 (7-36amide) are actually mediated via the DPP-4-generated GLP-1 (9-36amide) and its smaller degradation products and, thus, are independent of GLP-1R signaling. At a dose of 0.3 nmol/L, pretreatment with GLP-1 (7-36amide) improves recovery of LVDP after ischemia/reperfusion (I/R) injury in both WT and GLP-1R KO mice [201]. Interestingly, a >10 fold greater dose of exendin-4 is needed relative to GLP-1 (7-36amide) to achieve a similar effect in WT mice but still with lower efficacy relative to GLP-1 (7-36amide) in GLP-1R KO mice [201]. The superior cardioprotective effect of GLP-1 (7-36amide) relative to exendin-4 and the preservation of efficacy of GLP-1 (7-36amide) but not exendin-4 in GLP-1R KO mice suggest that some of the cardioprotective effects of GLP-1 (7-36amide) are independent of GLP-1R signaling and are potentially mediated via the DPP-4-cleaved GLP-1 metabolite GLP-1 (9-36amide) [201]. Interestingly, when mice are pretreated with either GLP-1 (7-36amide) or GLP-1 (9-36amide), only GLP-1 (7-36amide) improves LVDP. However, when administered during reperfusion, both GLP-1 (7-36amide) and GLP-1 (9-36amide) improve functional recovery from I/R injury in both WT and GLP-1R KO mice [201]. Consistent with these data, while exendin-4 but not GLP-1 (9—36) has infarct limiting potential in the isolated rat heart, exendin-4 and GLP-1 (9—36) both improve left ventricular performance during reperfusion [626]. The infarct-limiting potential of exendin-4 is abolished by treatment with exendin (9—39), thus indicating that this cardioprotective effect is GLP-1R-dependent [626]. Treatment of isolated mouse cardiomyocytes with either exendin4 or GLP-1 (9-36amide) increases phosphorylation of AKT and ERK [631], both of which are known to positively affect cardiomyocyte growth and survival [632,633].

Collectively, these data indicate that pharmacologic administration of GLP-1 (9-36amide) may have a functional role to improve recovery from I/R injury via GLP-1R-independent mechanisms, whereas GLP-1 (7-36amide) but not GLP-1 (9-36amide) affects cardiac contractility via GLP-1R signaling [201]. Further supporting a cardiometabolic role of GLP-1 (9-36amide), both GLP-1 (7-36amide) and GLP-1 (9-36amide), but not exendin-4, promote vasodilation in isolated and *ex vivo* cultured mesenteric blood vessels [201], i.e., the vasodilatory effect of GLP-1 (7-36amide) was not different relative to that of GLP-1 (9-36amide) and was likewise preserved in GLP-1R KO mice [201]. Whether the low endogenous concentration and the restricted pharmacokinetic action profile of endogenous GLP-1 metabolites are sufficient to elicit such effects under physiological circumstances remains questionable.

12.3. Effect of GLP-1 on heart rate (HR) and blood pressure (BP)
GLP-1 effects on HR and BP are species-related. In rats, intravenous infusion of GLP-1 (7-37amide) or exendin-4 acutely increases HR as well as systolic, diastolic, and mean arterial BP (Figure 7) [634,635]. In rats, GLP-1 stimulation of BP peaks around 3—5 min post-injection and returns to baseline after 25 min [634]. Both central and peripheral administration of GLP-1R agonists increase HR and BP in rodents [636—638], with induction of c-Fos expression in adrenal medullary catecholamine neurons and activation of tyrosine hydroxylase, the key enzyme involved in production of norepinephrine, in the brainstem [638]. These data collectively suggest that some of the acute cardiovascular effects of GLP-1 might be due to brain enhancement of catecholamine outflow and elevation of sympathetic tone. However, in rats, GLP-1 elevation of BP is preserved upon pretreatment with reserpine, propanolol or phentolamine, thus indicating that the hypertensive effect of GLP-1 does not exclusively depend on catecholamine signaling [634]. However, while the hypertensive effect of GLP-1 is not abolished by inhibition of β-adrenergic signaling [634], exendin-4 induction of HR is blocked by the β-adrenergic receptor antagonist propranolol [639], and exendin-4 fails to affect HR in adrenalectomized rats (as reviewed in [622]). In mice, both acute and chronic central administration of exendin-4 increases HR and BP with concomitant decrease in excitatory glutamatergic and inhibitory glycinergic neurotransmission to preganglionic parasympathetic cardiac vagal neurons. GLP-1 stimulation of HR and BP is mediated via GLP-1R signaling because i.v. infusion of exendin (9—39) blocks GLP-1 and exendin-4 induction of HR and BP [635], and these actions are lost in *Glp1r$^{-/-}$* mice [640]. Collectively, these data suggest that acute GLP-1 effects on HR and BP in normal animals may involve signaling via both, the sympathetic and parasympathetic nervous systems. Not all rodent studies report elevated BP in response to GLP-1R agonism, but dose could be a discriminatory factor. While some studies report increased BP in rodents upon acute or chronic GLP-1R agonism [634—636,638], others report decreased BP, especially in experimental models associated with the development of hypertension such as salt-sensitive db/

db mice and Dahl S rats [641,642]. In mice, liraglutide blunts hypertension induced by angiotensin II (Ang-II), an effect that is absent in GLP-1R KO mice, and is also attenuated upon pretreatment of WT mice with exendin (9–39) [640].

While in most rodent studies GLP-1 increases HR and BP, i.v. infusion of GLP-1 (7-36amide) acutely increases HR without affecting mean arterial BP in conscious calves [643]. Most human studies report a moderate stimulatory effect of GLP-1 on HR with unchanged or with reduced BP only in hypertensive individuals [620,622,640,644–649]. Acute infusion of GLP-1 increases heart rate and cardiac output in healthy human volunteers, potentially via GLP-1-induced vasodilation in adipose tissue and skeletal muscle [650]. As demonstrated in healthy human volunteers, GLP-1 infusion acutely increases blood flow in the subcutaneous abdominal adipose tissue and skeletal muscle without alterations in splanchnic blood flow [650]. Notably, in contrast to GIP [651–654], the GLP-1 induced increase in adipose tissue blood flow is not dependent on postprandial hyperglycemia and hyperinsulinemia and is observed even under conditions of fasting glucose and insulin concentrations [650].

GLP-1R agonism has also been demonstrated to reduce BP in people with T2DM (as reviewed in [611,622,649]). Notably, in healthy human volunteers, acute administration of GLP-1, GLP-1 (9-36amide), or exenatide did not affect blood flow in the mesenteric or renal arteries, excluding splanchnic vasodilatation as a potential mechanism underlying the chronic hypotensive effect of GLP-1 [644]. In a recent meta-analysis of 60 clinical studies, GLP-1R agonists were demonstrated to reduce diastolic BP with a range of −1.84 to −4.60 mmHg and to slightly increase heart rate by 2–3.35 beats/min [620]. Relative to placebo, the reduction of diastolic BP only reached significance for the treatment of exenatide 10 μg/twice daily (−1.08 mmHg) [620]. These data align with an integrated analysis of 36 clinical studies that affirmed cardiovascular safety with up to 4-years' treatment with either DPP-4 inhibitors or GLP-1R agonists [655].

The molecular mechanisms underlying GLP-1 improvement of BP are incompletely understood. Potential mechanisms may include GLP-1-induced vasodilation and activation of nitric-oxide-dependent mechanisms [612,640,656]. Indeed, liraglutide stimulates relaxation of the aorta [640] but GLP-1 might also indirectly lower BP via its ability to reduce body weight. GLP-1R agonism could also improve BP via its ability to stimulate sodium excretion (natriuresis) via the kidneys [657]. The long-term steady-state BP is affected by the intravascular volume, which is influenced by the vascular tone and the extracellular fluid volume (ECFV); the ECFV is in turn determined by the sodium balance. Accordingly, when BP increases, the kidneys respond by increasing natriuresis to decrease BP via reduction of the ECFV [92,658,659]. GLP-1R agonists acutely stimulate natriuresis in rodents and may also do so in humans, lowering BP, potentially also via reduction of the ECFV [641,642,660,661]. In mice, liraglutide reduces angiotensin II (Ang-II) stimulation of systolic and diastolic BP, an effect that is absent GLP-1R KO mice or by pretreatment with exendin (9–39) or the natriuretic peptide receptor antagonist anantin [640]. In WT mice and in isolated atrial cardiomyocytes, liraglutide stimulates the production and secretion of atrial natriuretic peptide (ANP), and liraglutide fails to induce natriuresis and to lower BP in ANP KO mice [640]. Liraglutide stimulation of cardiac ANP secretion is PKA independent but mediated by Epac2 in a cAMP and PLC-dependent manner [640]. However, in humans there is no consistent effect of GLP-1 on ANP secretion whereas angiotensin II is generally lowered [657,662–664]. In summary, GLP-1R agonism improves BP through stimulation of natriuresis via Epac2-dependent stimulation of cardiac ANP secretion [640]. Nevertheless, the

majority of hypertensive human subjects treated with liraglutide do not exhibit an increase in circulating ANP [662].

13. GLP-1 EFFECTS ON FOOD INTAKE AND BODY WEIGHT

One of the most frequently described extrapancreatic effects of pharmacokinetically-optimized GLP-1R analogs is their ability to lower body weight via centrally regulated inhibition of food intake (Figure 7). Suppression of food intake by GLP-1R agonism has been demonstrated in numerous species including mice [465–467,665,666], rats [667,668], birds [669,670], pigs [671,672], non-human primates [673,674], and humans [675–678]. The anorexigenic effect of GLP-1R agonism has been demonstrated in numerous clinical studies and fMRI analysis support that GLP-1 inhibition of food intake is centrally mediated in healthy individuals and patients with type-2 diabetes [679–684].

13.1. Effect of peripheral administration of GLP-1 analogs on food intake
Peripheral administration of GLP-1 analogs decreases body weight via suppression of food intake [465–467,665,685]. Depending on the molecule (native GLP-1 or a longer-acting GLP-1 analog) and the route of administration, GLP-1 and its analogs seem to engage different signaling pathways to either directly or indirectly transmit the signal to reduce food intake to the CNS.

Central GLP-1R appear to be necessary for the anorectic effect of peripherally administered GLP-1R agonists in rodents [686–688]. When administered s.c., liraglutide fails to inhibit eating in mice possessing a CNS-specific deletion of GLP-1R [688]. Another report indicates that i.v. infusion of liraglutide inhibits eating in rats by directly activating hypothalamic Pomc/Cart neurons in the ARC, without activating GLP-1-producing neurons in the hindbrain, and independent of GLP-1R signaling in vagal afferents, the area postrema (AP) and the PVN [686]. In rats, central (i.c.v.) administration of exendin (9–39) was also sufficient to attenuate the anorexigenic effect of i.p. injected liraglutide and exendin-4 [687], and bilateral neurochemical lesions of the lateral parabrachial nucleus (lPBN) with ibotenic acid blunted the anorexigenic effect of peripherally administered exendin-4 [689]. When applied either s.c. or i.p., exendin-4 and liraglutide induced acute neuronal activation (measured by activation of cFos) in PVN, AP, and NTS [686,690]. Interestingly, the short-term anorexigenic effects of i.p. injected low-dose liraglutide and exendin-4 were also attenuated by subdiaphragmatic vagal deafferentation [687,691], but longer-term effects of these compounds on food intake did not depend on intact vagal afferents [691]. All of these findings are consistent with the concept that GLP-1 receptor agonists inhibit eating and reduce body weight primarily by acting directly in the brain, and that various brain areas are involved in mediating the eating-inhibitory effects of GLP-1 receptor agonists. This presumably reflects the ability of long-acting GLP-1 analogs to enter the brain at areas with an incomplete BBB, such as the median eminence.

While these data collectively emphasize the importance of neuronal GLP-1R for the food intake inhibition by long-acting GLP-1 analogs, it is unclear whether and to what extend this accounts for physiological doses of intestinal-derived native GLP-1. When infused into the hepatic portal vein, GLP-1 (7-36amide) increased cFos expression in the NTS, the AP, and the central nucleus of the amygdala, but not in the hypothalamic ARC or PVN [692], i.e., native GLP-1 appears to induce an activation pattern in the brain that is different from the pattern induced by long-acting GLP-1 receptor agonists. While peripherally administered GLP-1 seemingly crosses the blood brain barrier (BBB) via simple

Review

diffusion, the half-life of GLP-1 is less than 2 min, leading to disagreement regarding whether peripherally derived GLP-1 is what activates CNS GLP-1R under physiological conditions.

In rats, i.v. administration of GLP-1 at high physiological doses stimulates vagal afferents, and this is blocked by pretreatment with exendin (9—39) [693]. While this activation appears to be involved in the insulinotropic and gastric emptying inhibiting effects of exogenous GLP-1 [693], it does not appear to be crucial for the effect of i.v. administered native GLP-1 on eating [694], because GLP-1 infusions into the hepatic portal vein did not lose their potency to reduce food intake after subdiaphragmatic vagal deafferentation in rats [692,694]. Interestingly, however, the food intake inhibition following i.p. administration of a supposedly "physiological" dose of GLP-1 (7-36amide) depends on the integrity of abdominal vagal afferents [694], indicating that peripheral GLP-1 can inhibit eating via at least two partly separate pathways. Consistent with a role of vagal afferents and vagal afferent GLP-1 receptors in the eating-inhibitory effect of peripheral endogenous GLP-1, virus-mediated knockdown of GLP-1R in vagal afferent neurons increased meal size, accelerated gastric emptying, and reduced prandial insulin secretion in rats, while not affecting 24 h food intake or body weight [518]. The latter finding matches the results obtained in Phox2b-Cre GLP-1 receptor KO mice, lacking GLP-1 receptors in the autonomic nervous system, which have normal body mass and composition in response to both chow and HFD [688], yet exhibit partial attenuation of the anorectic and weight loss effects of peripherally administered GLP-1R agonists [520]. In humans, the effect of i.v. GLP-1 infusion on food intake was lost after truncal vagotomy [695], indicating that vagal afferents are involved in mediating the effects of circulating native GLP-1 on appetite. In rats, on the other hand, lesions of the AP or administration of exendin (9—39) into the 4th ventricle blocked the food intake inhibition by GLP-1 infused into the hepatic portal vein [261], suggesting that high concentrations of circulating GLP-1 act on the hindbrain to reduce food intake. In one study GLP-1 infused into the vena cava or hepatic portal vein of rats had similar effects on food intake [696], whereas in another study in rats GLP-1 infusions into the jugular vein inhibited food intake more potently than infusions into the hepatic portal vein [697]. These findings argue against a hepatic portal site of action of GLP-1 to inhibit eating, and further emphasize that the route of administration is an important consideration when analyzing food intake inhibition by GLP-1R agonism.

The data summarized above were collected in mice, rats, and humans; in addition to varying experimental conditions, species differences may account for some of the discrepancies observed. Nevertheless, collectively these data indicate that vagal afferents are involved in mediating the satiating effects of endogenous peripheral GLP-1 or exogenously administered GLP-1 and the acute inhibition of food intake in response to peripheral GLP-1 receptor agonists under certain conditions. However, vagal afferents and vagal afferent GLP-1 receptors are presumably less important for the effects of peripheral GLP-1 on body mass.

13.2. Effect of central administration of GLP-1R analogs on food intake

The acute anorectic effect of centrally administered GLP-1 was described simultaneously 1996 by Steve Bloom's group [668] and by Mads Tang-Christensen et al. [92] and was confirmed by others [667,698]. In mice and rats, the short-term inhibition of food intake induced by i.c.v. administered GLP-1R agonists is dose-dependent, can be blocked by pretreatment with exendin (9—39) [668,698] and is absent in GLP-1R KO mice [690]. Inhibition of food intake by centrally

administered GLP-1R analogs is accompanied by increased cFos neuronal activity in the PVN, amygdala, NTS, AP, lateral parabrachial nucleus, and the ARC [668,696,698]. These data align with the expression of GLP-1R throughout the CNS [395,396]. Furthermore, cell bodies that are immunoreactive to GLP-1 antisera are detected in the NTS, and GLP-1 immunoreactive nerve fibers are widely distributed throughout the brain with highest density in the hypothalamus, thalamus and septal regions whereas the lowest density is in the cortex and hindbrain [86,698]. Supporting a role of the NTS in mediating GLP-1's effects on food intake, adeno-associated virus (AAV) mediated knock-down of GLP-1R in the NTS results in higher food intake and meal size [699]. Chronic blockade of GLP-1R with intra 3rd ventricular infusion of exendin (9—39) increases food intake and fat mass [700]. Direct intraparenchymal delivery of low, ventricle subthreshold doses of GLP1R agonists to GLP1R expressing nuclei throughout the brain, including e.g., NTS, lPBN, ventral tegmental area (VTA), PVH, PVT, nucleus accumbens (NAcc), ventral hippocampus, and lateral septum reduced food intake via reductions in meal size. Consistent with this, delivery of exendin (9—39) to these sites increases meal size and food intake [701—705].

Hindbrain GLP-1R activation enhances phosphorylation of PKA and MAPK while decreasing the activity of AMPK in the NTS, and inhibition of PKA/MAPK activity by administration of Rp-cAMP or UO126 attenuates food intake inhibition of exendin-4 administered into the 4th ventricle [706]. These data suggest that hindbrain GLP-1R activation suppresses food intake via PKA/MAPK-induced inhibition of AMPK. Diphtheria toxin-induced ablation of GLP-1 producing neurons in the NTS had no effect on either ad libitum food intake or glucose metabolism [90]. Nevertheless, in rats, activation of hindbrain GLP-1R via i.c.v. administration of exendin-4 into the 4th ventricle reduces food intake via reduction of meal frequency; i.e., via increased satiety [706]. Intra 4th-ventricular administration of exendin (9—39) or lesions of the AP also block food intake inhibition by GLP-1 infused into the hepatic portal vein (HPV) [261], suggesting that high levels of circulating GLP-1 inhibit eating at least in part by acting on the hindbrain.

13.3. Molecular mechanisms underlying food intake inhibition by GLP-1

The molecular underpinnings regulating GLP-1 inhibition of food intake and body weight loss are complex. Undoubtedly, brain GLP-1R signaling is crucial for the anorexigenic effect of exogenously administered long-acting GLP-1R agonists, as demonstrated by failure of liraglutide to suppress food intake in CNS-specific GLP-1R KO mice [688]. Consistent with this, i.c.v. administration of exendin (9—39) is sufficient to attenuate the anorexigenic effect of peripherally administered liraglutide and exendin-4 [687]. However, CNS-specific GLP-1R KO mice have normal body mass and composition whether on a chow or HFD [688], suggesting that these receptors may be necessary for the pharmacological response to GLP-1 but not the physiological control of body weight. However, data obtained in KO animals generally have to be regarded with caution since compensatory mechanisms, which limit interpretations and affect study outcomes, might develop. Depending on the route of administration (and the GLP-1R agonist used), peripherally administered GLP-1 analogs seem either to act directly in the hypothalamus and hindbrain, both areas with incomplete BBB, or else on vagal afferents to transmit the signal to the hindbrain which then projects to other key feeding areas in the brain. Hindbrain GLP-1R activation enhances phosphorylation of PKA and MAPK while decreasing the activity of AMPK in the NTS, and inhibition of PKA/MAPK activity by administration of Rp-cAMP or UO126 attenuates food intake inhibition of exendin-4 administered into the 4th ventricle [706]. These

data suggest that hindbrain GLP-1R activation suppresses food intake via PKA/MAPK-induced inhibition of AMPK.

The anorexigenic effect of GLP-1R agonism involves the glucose-dependent silencing of central AMPK action. Activity of AMPK is increased under conditions of fasting [707] and is decreased under conditions of enhanced glucose utilization [708—710]. Consistent with AMPK's role as a stress- and nutrient-responsive protein kinase, pharmacological, physiological (e.g. fasting-induced), or genetic activation of AMPK stimulates food intake whereas AMPK inhibition decreases food consumption [707,711]. Central administration of GLP-1 reduces the activity (phosphorylation) of AMPK [712,713] and treatment of mouse hypothalamic GT1-7 cells with exendin-4 stimulates glycolysis and decreases phosphorylation of AMPK [714]. Pharmacological activation of AMPK by administration of 5-aminoimidazole-4-carboxamide-1-β-ᴅ—ribofuranoside (AICAR) in either the 3rd [714] or the 4th ventricle [706] attenuates the anorectic effect of exendin-4 injected into the same ventricles. Consistent with these data, i.c.v. administration of the glycolysis inhibitor 2-deoxyglucose (2-DG) likewise increases activity of AMPK and blunts food intake suppression by i.c.v.-injected exendin-4 [714]. In summary, compelling evidence indicates that the anorexigenic effect of central GLP-1 receptor activation is partially mediated by central stimulation of glycolysis and concomitant silencing of AMPK activity. Accordingly, pharmacological activation of AMPK in the VMH blunts food intake suppression by i.c.v. exendin-4 [714,715] and attenuates central GLP-1 effects on brown and inguinal white adipose tissue [666].

The anorexigenic effect of GLP-1 seems to be transduced via GLP-1R signaling in several areas of the brain, including both hypothalamic nuclei and the hindbrain. Microinjection of exendin-4 into the NTS reduces intake of palatable high-fat diet and inhibits food reward behavior [716], and hindbrain inhibition of GLP-1R via administration of exendin (9—39) into the 4th ventricle increases food intake [717]. The anorexigenic and weight-lowering effects of liraglutide are blunted upon genetic ablation of GLP-1R in glutamatergic neurons, yet fully preserved when GLP-1R is deleted in GABAergic neurons [718]. Peripheral administration of GLP-1 (7-36amide) enhances neuronal activity in the VMH [719,720], and direct administration of GLP-1 into the LH, VMH, or DMH of rats acutely decreases food intake [721]. Similar findings were reported in rats that received direct infusion of GLP-1 (7-36amide) into the PVN [722,723]. In rats, liraglutide decreases food intake when directly injected into the ARC, LHA, or PVN while direct administration into the VMH decreases body weight without affecting food intake, but increases the expression of *Ucp1* in the brown and inguinal white adipose tissue [666]. The acute effects of exendin-4 to reduce food intake and to improve glucose handling are preserved upon knockdown of GLP-1R in the VMH [715], indicating that the effects of central GLP-1R activation on eating and BAT thermogenesis are mediated by at least partially separate brain areas and circuitries.

13.4. GLP-1 effects on visceral illness

Inhibition of food intake can be due to other factors than satiety, including gastric discomfort, e.g., gastroparesis, associated with visceral illness and stress. Anorexia associated with visceral illness can be chemically-induced by administration of lithium chloride (LiCl), a substance known to promote nausea and emesis in humans [724—726] and taste avoidance conditioning and pica in rodents, that do not vomit [727]. Pica is the measure of the amount of kaolin clay consumed in response to treatments that trigger nausea and vomiting in humans and is a broadly accepted proxy of visceral malaise in non-vomiting rats. Taste avoidance conditioning describes the reduction in the intake of novel taste or food that has been associated with a treatment that induces negative consequences e.g., including the effects of peripheral pain, lactose malabsorption or the effects LiCl [728]. In rats, peripheral administration of LiCl induces cFos neuronal activity in the lPBN, the AP and the NTS [726,729] with remarkable overlap in the cFos activity pattern induced by centrally administered GLP-1 [726]. Apart from inducing cFos activity in the same brain areas [696,726,730], LiCl and central GLP-1R agonism both decrease locomotor activity [668,731], slow GI motility and gastric emptying [573,732], reduce body temperature [732,733], and induce adverse gastrointestinal effects including nausea, emesis, and taste avoidance [696,724—726,734—737]. Selective ablation of NTS PPG neurons prevents stress-induced hyperphagia in mice but has no effect on long-term ad libitum food intake, body weight, or glucose tolerance [90]. These data suggest that NTS PPG neurons are not essential for long-term regulation of food intake but are required for the short-term hypophagia in response to stress [738,739].

Collectively, these studies highlight that, in some species, GLP-1 and LiCl might engage similar central signaling mechanisms and suggest that central GLP-1s anorexigenic effect might be influenced by aversive pathways and gastric discomfort. However, there are other data that would challenge the simple explanation that the reduction in feeding and body weight resulting from CNS GLP1R agonism is simply explained by inducing an aversive visceral state. Targeted agonist delivery to CNS GLP1-R expressing sites, PVT, NTS, VTA, NAcc, or PBN reduces food intake without the expression of pica, a proxy of visceral illness [704,716,740,741]. In addition, Jelsing et al. report that food intake inhibition by liraglutide is observed in the absence of reduced gastric emptying effects that some have linked to visceral malaise [742]. Importantly, both van Bloomendaal et al. [682] and ten Kulve et al. [743] provide data to support the hypothesis that food intake reduction after GLP-1 receptor agonism is mediated by actions on appetite- and reward-related areas of the human brain, an interpretation also supported by rodent studies [704,716,740,741].

13.5. GLP-1 effects on reward behavior

Central GLP-1 regulation of food intake is not restricted to GLP-1R signaling in the hypothalamus and hindbrain and is not limited to modulation of homeostatic feeding, i.e. food intake to maintain energy homeostasis. GLP-1 also affects non-hunger related (hedonic) feeding by targeting brain areas involved in reward, motivation and addiction, such as the VTA, NAcc, lateral septum [701,741,744,745], and PVT [704]. These mesolimbic regions express GLP-1R [347] and receive projections from PPG neurons in the NTS [354,741,744]. Peripheral administration of exendin-4 [691] or direct administration of GLP-1 into the core of the NAcc [744] increases cFos expression in this region, and direct activation of NAcc GLP-1R reduces food intake while its direct inhibition by exendin (9—39) induces hyperphagia [744]. Food intake inhibition by NAcc GLP-1R is unrelated to taste avoidance [744] and does not induce pica [701,741]. Administration of exendin-4 into the shell of the NAcc decreases sucrose reward behavior in the progressive ratio (PR) operant conditioning test [701]. Consistent with this, GLP-1R agonism decreases a variety of reward behaviors in rodents, including the motivation to lever press for food reward in an operant conditioning test [746], alcohol intake [747,748], alcohol seeking behavior [747], amphetamine-induced conditioned place preference (Amp-CPP) [748], and hedonic feeding [748]. The inhibitory effect of GLP-1R analogs on reward behaviors is blunted in mice with CNS-specific deletion of GLP-1R [748]. Several lines of evidence indicate that NAcc GLP-1R activation decreases food intake by reducing food palatability. In satiated rats given a choice between chow and high-fat diet (HFD), exendin-4 preferentially reduces consumption of the more

Review

palatable HFD [741]. In rats, intra-NAcc injection of exendin (9—39) increases the size of a sucrose meal and accelerates the licking frequency during the first meal, an indicator of increased palatability [749]. In summary, GLP-1R agonism decreases homeostatic and hedonic feeding, and modulates food intake not only via the hypothalamus and the hindbrain but also via signaling in the mesolimbic system, in which GLP-1R activation affects reward behavior and palatability.

13.6. GLP-1 transport across the blood brain barrier (BBB)

Several lines of evidence indicate that peripherally administered GLP-1 can cross the blood brain barrier (BBB). Studies using a stabilized radiolabeled (Ser8)GLP-1 revealed rapid uptake into the brain after intravenous (i.v.) administration without self-inhibition when challenged with increased doses of unlabeled (Ser8)GLP-1 or upon inhibition of brain GLP-1 influx upon pretreatment with exendin (9—39). Small peptide GLP-1R analogs including liraglutide, lixisenatide, and exendin-4 have all been demonstrated to cross the BBB upon peripheral administration [750,751], and HPLC analysis confirmed that most of the peripherally administered exendin-4 reaches the brain intact [751,752]. While these studies suggest that GLP-1 influx into the brain might be a GLP-1R-independent process with passive diffusion of GLP-1 through the BBB [750—752], other studies suggest that uptake of liraglutide into the brain is GLP-1R-dependent [686,753]. Nonetheless, brain regions with incomplete BBB, such as the subfornical region and the AP, are main targets of peripherally injected 125I-labeled GLP-1 [754].

14. GLP-1 EFFECTS ON WATER INTAKE

GLP-1 inhibition of water intake was first reported by Mads Tang-Christensen in 1996 [92] and was later confirmed by others [755—760]. GLP-1 inhibition of water consumption is independent of food intake [758] and is blocked by pretreatment with exendin (9—39) [92]. In rats, inhibition of drinking by GLP-1 (7—36 amide) is dose-dependent and occurs within 30 min following its central (i.c.v.) or peripheral (i.p.) administration [92]. In one study in healthy humans, i.v. infusion of GLP-1 decreased water consumption by 36% in response to i.v. infusion of hypertonic saline (5% NaCl) [755]. Peripheral but not central administration of GLP-1 fails to inhibit angiotensin (ANG) II-induced drinking in rats that have been treated with monosodium glutamate (MSG) to induce damage of the ARC and the sensory circumventricular organs, suggesting that peripherally administered GLP-1 effects drinking behavior via CNS-dependent mechanisms [760].

GLP-1 affects renal function by decreasing water intake as well as by stimulating the excretion of urine and sodium (Figure 7). The natriuretic and diuretic effect of GLP-1R agonism was first shown in 1996 [92] and was subsequently confirmed in rodents [642,660,761] and in healthy, obese, and type-2 diabetic humans [661,663]. While the acute natriuretic effect of GLP-1 has been solidly confirmed in humans [762—764], 12 wk treatment of T2DM patients with liraglutide or the DPP-4 inhibitor sitagliptin had no effect on markers of renal function, including glomerular filtration rate, renal plasma flow, albumin-to-creatine ratio, and excretion of sodium, potassium, and urea [765]. These data suggest that that GLP-1's effects on renal function might be transient and vanish upon more chronic treatment. In one study in T2DM patients, the natriuretic effect of liraglutide persisted after 3 wk treatment [662].

In rats, GLP-1 induced natriuresis and diuresis is accompanied by increased renal plasma flow and glomerular filtration rate [660,761]. Denervation of the kidney results in failure of GLP-1 to increase glomerular filtration rate and decreases the natriuretic and diuretic effect of GLP-1 [761]. These data suggest that the natriuretic and diuretic effect of GLP-1 might be due to inhibition of sodium reabsorption in the proximal tubule and emphasize that GLP-1 requires intact renal innervation to increase glomerular filtration. Studies in rodents and humans indicate that GLP-1R is expressed in the renal vasculature [392,766—768]. However, treatment of isolated porcine proximal tubular cells with GLP-1 reduced sodium re-absorption [768]. Consistent with this, GLP-1, exendin-4, and the DPP-4 inhibitor sitagliptin reduced proximal tubular sodium reabsorption in rats [660,761,769,770] and humans [663]. In the renal proximal tubule, GLP-1 reduced Na^+/H^+ exchanger isoform 3 (NHE3)-mediated bicarbonate reabsorption [660]. Consistent with this, exendin-4 decreased NHE3-mediated sodium-dependent pH recovery in porcine kidney LLC-PK$_1$ cells [771]. Inhibition of NHE3 activity by GLP-1R agonism seems to be mediated via cAMP activation of PKA, which phosphorylates NHE3 at its Ser552 and Ser605 residues [660,771,772]. In rodents [660,761] and humans [663,763], GLP-1R agonism increases renal lithium clearance, a marker for proximal tubular sodium reabsorption. These data suggest that the diuretic and natriuretic effects of GLP-1 include changes in renal hemodynamics and reduced sodium reabsorption due to PKA-dependent down-regulation of NHE3 activity in the renal proximal tubule. GLP-1 might also affect kidney function via its cardiovascular effects. In rats, infusion of GLP-1 increases renal blood flow and the glomerular filtration rate [660,761], and this effect is potentially mediated via GLP-1's ability to increase vasodilation of the afferent renal arteriole. In wildtype but not GLP-1R KO mice, liraglutide stimulates the secretion of natriuretic peptide (ANP) from the atrial cardiomyocytes and liraglutide fails to affect natriuresis and vasorelaxation in mice deficient for ANP [640].

15. GLP-1 EFFECTS ON ENERGY EXPENDITURE

A role of endogenous GLP-1R signaling in the control of energy expenditure was inferred from studying mice with congenital GLP-1R deficiency. Relative to wildtype controls, lean chow-fed GLP-1R KO mice do not have alterations in energy expenditure, neither when kept at a standard room temperature nor when challenged with moderate cold exposure [773]. However, relative to wildtype controls, diet-induced obese GLP-1R KO mice exhibit reduced energy expenditure at standard ambient room temperature but not at temperatures favoring thermoneutrality [774]. Because thermoneutrality minimizes the contribution of non-shivering thermogenesis to whole-body energy expenditure and because non-shivering thermogenesis requires functional brown adipose tissue (BAT) [775], these data imply a contribution of endogenous GLP-1R signaling to the control of energy expenditure, at least in diet-induced obese mice, by regulating BAT activity. This contribution requires GLP-1R signaling in the brain; i.e., the brain plays a critical role in the control of BAT activity [776] and GLP-1R is expressed in the brain, including in nuclei involved in the control of BAT [347,389]. In keeping with this, site-specific disruption of GLP-1R on neurons of the DMH of rats results in significant body weight gain paralleled by a reduction in energy expenditure and BAT heat production [777]. The DMH is part of the neuronal network involved in the control of BAT thermogenesis [776] and receives innervation from pre-proglucagon-expressing neurons of the hindbrain [778]. *A priori*, this positions those neurons as potential contributors to the neural circuits controlling energy expenditure by regulating BAT thermogenesis in rodents. Intriguingly, emerging data involving gain or loss of function from those cells in mice argue against such a

contribution. Thus, diphtheria toxin-mediated ablation of those neurons [90] does not result in long-term changes on food intake or body weight, which suggests a minimal impact on energy expenditure. Conversely, acute chemogenetic activation of those neurons fails to increase, and rather transiently reduces, energy expenditure [357]. Disregarding differences due to experimental conditions including acute versus chronic intervention or animal species or strains, these data suggest that endogenous GLP-1R signaling in the brain may play a constitutive role in the homeostatic control of BAT thermogenesis, beyond its physiological modulation by brain-derived GLP-1.

However, this does not preclude the possibility that exogenous administration of GLP-1 may regulate energy expenditure by acting on brain GLP-1R. In fact, an early report in rats described an increase in energy expenditure during the first hours of the dark cycle following i.c.v. administration of low doses of GLP-1 (7-37amide) [779].

In mice, acute central (i.c.v.) but not peripheral administration of GLP-1 increased brown adipose tissue (BAT) temperature and increased the activity of sympathetic fibers that innervate the inguinal BAT [773]. Consistent with these data, central administration of the GLP-1R agonists liraglutide and exendin-4 increases energy expenditure and BAT thermogenesis through mechanisms that include AMPK signaling in the mediobasal hypothalamus [666]. Taken together, these studies suggest that pharmacological activation of brain GLP-1R signaling increases energy expenditure in mice.

Peripheral administration of GLP-1R agonists has led to variable results with respect to energy expenditure. One study in rats found an increase of energy expenditure upon i.v. infusion of GLP-1, and with even greater potency relative to centrally administered GLP-1 [780]. These data are consistent with the demonstration that i.v. but not i.p. administered GLP-1 inhibits eating via activation of hind-brain GLP-1 receptors [261,694] and suggests a similar mechanism for i.v. GLP-1-stimulation of energy expenditure. In contrast, another study in rats reported an acute decrease of energy expenditure associated with a decrease in interscapular skin tem-perature following i.p. administration of exendin-4 and this effect was attenuated when GLP-1R was knocked down on vagal afferent neurons [781]. These data are consistent with reports that the anorexigenic effect of i.p. administered GLP-1 depends on vagal afferents [692,694] and thus suggests a similar mechanism for i.p. GLP-1 effects on energy expenditure. Caudal brainstem delivery of Cocaine- and amphetamine-regulated transcript (CART) induces hypothermia, and this effect is blocked by pretreatment with exendin (9—39) [782]. Several studies in mice [465,467,685,774] and humans [783—787] have failed to detect changes in energy expenditure following chronic or acute peripheral treatment with different GLP-1R agonists. One human study did report an acute decrease of diet-induced thermogenesis due to lower carbohydrate oxidation following i.v. infusion of GLP-1 [788].

In summary, the experimental evidence supports a contribution of brain-GLP-1R signaling in the control of energy expenditure, at least in rodents, via control of BAT activity. The frequently reported absence of peripherally administered GLP-1 mimetics to regulate energy expen-diture in mice and humans has several implications. First, the BBB might limit access to brain areas where GLP-1R could potentially mediate effects on energy expenditure; second, the underlying GLP-1 analog, the feeding status of the test subject, and the route of administration are important considerations when analyzing GLP-1 effects on energy expenditure, and, third, rodents may be uniquely sensitive to mechanisms activating energy expenditure through the sympathetic nervous system.

16. GLP-1 EFFECTS ON GASTRIC EMPTYING

In addition to its direct and vagally mediated insulinotropic action on β-cells, GLP-1 also improves postprandial glucose handling by inhibition of gastric emptying, decelerating the rate at which glucose is absorbed into the circulation (Figure 7) [573,789—791]. Inhibition of gastric motility and gastric acid secretion following treatment with GLP-1R agonists has been demonstrated in mice [792], rats [793—795], dogs [796—798], pigs [799,800], and humans [573,789—791,799,801—805]. In rats, inhibition of gastric emptying occurs af-ter peripheral or central administration of GLP-1 [795], and this effect is mediated via hindbrain GLP-1R signaling [806]. Vagal afferent GLP-1 receptor knockdown accelerates gastric emptying [518] and vagal afferent denervation [795] or peripheral administration of exendin (9—39) [794,795] blocks the effect of centrally or peripherally adminis-tered GLP-1 on gastric emptying. These data are consistent with the failure of GLP-1 to inhibit gastric acid secretion in vagotomized human subjects [807] as well as the failure of GLP-1 to inhibit vagally-induced motility in the isolated perfused pig antrum [800]. These data suggest that GLP-1-mediated inhibition of gastric motility includes vagal af-ferents, as well as GLP-1R dependency in the periphery and the brain [795]. Inhibition of adrenergic signaling through combined adminis-tration of phentolamine and propanolol abolishes the inhibitory effect of GLP-1 on gastric motility, suggesting that GLP-1 inhibits gastric emptying via adrenergic signaling [794]. In fasted rats, co-administration of GLP-1 and GLP-2 leads to a greater inhibition of small bowel motility relative to either monotherapy [793]. Exendin-4 fails to affect gastric emptying in GLP-1R KO mice [792]. In healthy human volunteers, intravenous GLP-1 decelerates gastric emptying after the first meal to a greater extent than after a second meal, indicating that GLP-1 inhibition of gastric emptying undergoes rapid desensitization [803]. In overweight humans, liraglutide 3 mg solidly inhibits gastric emptying after 5wk of treatment, but the efficacy markedly decreases after 16wk of treatment, yet with still significant effect relative to placebo treated controls [808]. In rats, liraglutide- but not exendin-4-induced inhibition of gastric emptying is markedly diminished after 14 wk of treatment [742]. Because gastric emptying is a prerequisite for glycemic rises after meals, which in turn trigger insulin secretion, deceleration of gastric emptying in response to exogenous GLP-1 leads to lesser post-meal glucose excursions and reduced insulin secretory responses [809]. This has led to doubts about the importance of an incretin role for pharmacological GLP-1 administration, because incretin hormones should lead to a net in-crease in post-nutrient insulin secretory responses [810].

17. GLP-1 EFFECTS ON THE HYPOTHALAMIC PITUITARY ADRENAL (HPA) AXIS

Neurons of the NTS are important in the regulation of the hypothalamus-pituitary-adrenal (HPA) axis in response to stress [811], and evidence indicates that brain GLP-1R signaling plays an integral role in the acute CNS response to stress and aversive stimuli [811]. Preproglucagon-positive neurons of the NTS have dense projections to other brain areas, including the hypothalamic PVN, where they innervate CRH-releasing neurons [812]. GLP-1R is expressed in neu-rons of the PVN [347] that co-localize with CRH [813]. Central administration of GLP-1 in rats and mice stimulates the HPA axis and increases secretion of corticosterone, and this is accompanied by increased cFOS immunoreactivity in CRH-positive neurons in the PVN [668,698,814]. In rodents and humans, peripherally administered

Review

GLP-1R agonists transiently stimulate the HPA axis and increase circulating levels of corticosterone, aldosterone, and ACTH [815—817]. Peripheral administration of exendin-4 or liraglutide increase circulating corticosterone in mice under basal conditions and also during a forced swimming test [818]. Mice with PVN-selective deletion of GLP-1R (using Sim1 cre) have impaired stress-induced HPA axis activation, and this is further accompanied by protection from stress-induced weight loss, reduced stress-induced cardiovascular endpoints and reduced anxiety-like behavior [813]. In rats, combined administration of dexamethasone and exendin-4 leads to a greater anorexigenic effect and greater weight loss relative to treatment with exendin-4 alone [819]. Similar results were reported in mice treated with GLP-1/dexamethasone, an unimolecular peptide-nuclear hormone conjugate designed to optimize dexamethasone delivery into GLP-1R positive cells [820]. Ablation of hindbrain catecholamine neurons using anti-dopamine-β-hydroxylase-saporin (DSAP) blunts the ability of exendin-4 to increase corticosterone secretion but interestingly potentiates the food intake inhibition by exendin-4 [819]. These data suggest that activation of the HPA axis antagonizes the satiation effect of GLP-1R agonism [819].

18. GLP-1 EFFECTS ON LEARNING, MEMORY AND NEUROPROTECTION

GLP-1R expression has also been demonstrated in the rat [347,821] and mouse [822] hippocampus, a brain region implicated in spatial learning and memory [823,824]. In rats, central GLP-1R agonism improves several aspects of learning and memory, as demonstrated by improved performance in the Morris water maze test and by enhanced latency in the passive avoidance test [417]. GLP-1's improvement of learning and memory can be blocked by pretreatment with exendin (9—39), and is absent in mice deficient for the GLP-1 receptor [417]. Targeted hippocampal restoration of GLP-1R expression in otherwise GLP-1R-deficient mice using AAV-mediated gene transfer reverses GLP-1 improvement of learning and memory in the passive avoidance test [417].

Central GLP-1R signaling has also been demonstrated to have neuroprotective effects. GLP-1 and exendin-4 enhance differentiation and neurite outgrowth in rat pheochromocytoma (PC12) cells and in human neuroblastoma SK-N-SH cells [825]. The neuroprotective effect of GLP-1R agonism is similar to that conferred by nerve growth factor (NGF) and is blocked when PC12 cells are co-incubated with exendin (9—39) [825]. Both GLP-1 and exendin-4 protect cultured hippocampal neurons against glutamate-induced apoptosis [826]. The time and severity of seizures induced by systemic administration of the neurotoxin kainic acid is enhanced in GLP-1R KO mice relative to WT controls whereas the destructive effect of kainic acid is ameliorated in the GLP-1R KO mice upon targeted AAV-mediated restoration of hippocampal GLP-1R expression [417].

The molecular mechanisms underlying the neuroprotective effects of central GLP-1R agonism are, at least in part, mediated through the ability to increase cAMP formation and subsequently to enhance activation of PI3-kinase and ERK. GLP-1R agonism also increases cAMP levels in cultured hippocampal neurons [826] and in PC12 cells [825]. Pharmacological inhibition of either PI3-Kinase or ERK blocks the stimulatory effect GLP-1 and exendin-4 on neurite outgrowth in PC12 cells [825]. In PC12 cells, GLP-1 stimulation of neurite outgrowth is only partially silenced by treatment with the PKA inhibitor H89, indicating that cAMP-mediated activation of PI3K and ERK following GLP-1R agonism does not fully depend on PKA signaling, at least in these cells [825,827].

Huntington's disease (HD) is characterized by severe neurodegeneration that is caused by a mutation of the huntingtin protein (HTT) [828]. The neurotoxicity and neurodegeneration induced by the mutant HTT protein has been attributed to enhanced oxidative stress and accumulation of HTT due to inappropriate autophagic HTT clearance [829]. The prevalence of T2DM is elevated in patients with HD [830], suggesting that impaired insulin sensitivity might be a causal factor contributing to neurodegeneration in HD patients [830,831]. Overexpression of mutant HTT impairs insulin signaling and stimulates neuronal apoptosis in human SK-N-MC neuronal cells [831]. Treatment of SK-N-MC cells with liraglutide improves insulin sensitivity and enhances cell viability, potentially by mechanisms that include amelioration of neuronal glucotoxicity, improvement of oxidative stress and less aggregation of the mutant HTT through stimulation of AMPK-mediated autophagy [831].

Alzheimer's disease (AD) is characterized by neurodegeneration of cholinergic neurons in the hippocampus. In a rat model of neurodegeneration, GLP-1 and exendin-4 ameliorated neurodegeneration by reducing ibotenic acid-induced depletion of cholinergic cell bodies, as demonstrated by preservation of choline acetyltransferase immunoreactivity in basal forebrain cholinergic neurons [826]. In rats, administration of GLP-1R agonists into the hippocampus further prevented impairment of spatial learning and memory induced by central administration of amyloid β (Aβ), a protein considered to play a causative role in impairment of learning and memory in Alzheimer disease [832,833]. Consistent with this, chronic GLP-1R agonism has been demonstrated in several mouse models of AD to prevent the development of memory deficits [834—836]. Clinical studies evaluating the effect of GLP-1R agonism on neurodegeneration associated with AD are scarce. AD is frequently reported to be accompanied by decreased glucose transport across the BBB and 6 month treatment of AD patients with liraglutide improved BBB glucose transfer relative to placebo controls [837]. In a double blind placebo controlled study, 12 week treatment with liraglutide in patients at risk for AD did not show cognitive differences between the study cohorts [838]. Other clinical trials evaluating the neuroprotective effects of GLP-1R agonism on AD are currently ongoing [839]

GLP-1 analogs have also proven successful for the treatment of Parkinson's Disease (PD). PD is typically characterized by degeneration of dopaminergic neurons, a condition that can be mimicked in experimental animals by administration of the dopaminergic neurotoxin MPTP (1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridin) [840—842]. In mice, 7-d infusion of exendin-4 into the lateral ventricle was sufficient to protect from MPTP-induced damage of the dopaminergic system and from the development of locomotor deficits that typically arise from dopamine deficiency [843]. Similar neuroprotective effects have been reported in various PD rodent models treated with GLP-1R agonists [844—849]. Consistent with this, exendin-4 increases survival and levels of tyrosine hydroxylase, the key enzyme for dopamine production, in primary neuronal cultures treated with the dopaminergic toxin 6-hydroxydopamine (6-OHDA) [843]. Several clinical studies have confirmed the potential of GLP-1R agonism to ameliorate clinical symptoms of PD, including long-lasting improvements of motor and cognitive function [850—853].

19. GLP-1 EFFECTS ON BONE

The first understanding of GLP-1 actions in skeletal physiology arose from *GLP-1R* KO mice, which exhibit a reduction in trabecular bone mass due to a higher osteoclast activity [854]. In rodents, but not in humans, activation of thyroid GLP-1R leads to C-cell stimulation in the

thyroid [855,856], and involvement of calcitonin in GLP-1-mediated osteoclast activation has been suspected [854]. Furthermore, these animals exhibit profound modifications of bone matrix composition with a lower collagen maturity that jeopardizes the biomechanical response and hence contributes to skeletal fragility [857]. In comparison, *GIPR* KO animals present higher trabecular bone mass but significant lower biomechanical resistance due to alterations of tissue mineral density distribution and collagen maturity [858,859]. Double incretin receptor knock-out (DIRKO) animals revealed that the GLP-1/ GLP-1R pathway was important for the integrity of cortical but not trabecular bone [860].

GLP-1R agonists have been extensively used in animal models of skeletal fragility. In ovariectomy-induced bone loss, exenatide enhances bone formation due to osteoblast activation [857]. In hindlimb-unloading rats, exenatide improves bone mass by promoting osteogenic differentiation of bone marrow stem cells [861]. In type-1 diabetic models, although liraglutide is less effective than GIP analogues in promoting osteoblast differentiation, it improves tissue material biomechanics by reducing collagen degradation [862]. In type-2 diabetic models (genetically-inherited or diet-induced obesity), exenatide significantly enhances bone strength by increasing trabecular bone mass, bone formation and trabecular microarchitecture. Exenatide significantly improves collagen maturity [863,864]. The use of the unimolecular triple agonist GIP-Oxm is also significantly potent in improving bone strength and restoring trabecular bone mass [865]. However, its mechanism of action is linked to reduced bone formation and reduced osteoclast surfaces. GIP-Oxm leads to changes in collagen lamellar orientation, improves collagen maturity and reduction in advanced glycation end-products in the bone matrix. However, it is not clear whether these ameliorations in bone strength result from activation of skeletal GLP-1 receptors or whether it requires extraskeletal receptors as observed for the GIP/GIPR pathway [866]. As such, a stable GIP agonist has been demonstrated to improve bone quality in rats [867], whereas DPP-4 inhibition positively modified bone composition but not bone microstructure in high fat fed mice [868]. Human studies report less compelling data on the effects of GLP-1 or its analogues in bone remodeling markers or fracture. In contrast to GLP-2 administration, subcutaneous injection of GLP-1 in healthy individuals had no effects on bone formation or resorption markers [869]. In non-diabetic, overweight men, GLP-1 infusion seems to reduce the extent of bone resorption in a similar way to GIP [787]. However, in obese, non-diabetic patients under anti-psychotic drugs, exenatide failed to modify significantly the pattern of bone remodeling [870]. In contrast, liraglutide was capable of enhancing bone formation in obese weight-reducing women while having no effects on bone resorption [871]. Data are more controversial in type-2 diabetes and although first reports did not find evidence of protective effects of the GLP-1 receptor agonist on bone fracture [872,873], more recent data suggest that liraglutide and lixisenatide, but not exenatide, contribute to reduce fractures in the diabetic population [874].

20. PHARMACOLOGICAL USE OF GLP-1 ANALOGS TO TREAT OBESITY AND DIABETES

The exogenous supplementation of native hormones, commonly purified from tissue homogenates, has led to numerous, seminal discoveries, most prominently the identification of insulin in 1921 [875] followed by the discovery of glucagon in 1923 [3]. Despite numerous such groundbreaking discoveries, including identifying the intestine as a source of incretins [56], this practice did not necessarily identify hormones capable of sufficiently lowering body weight.

Its anorexigenic and insulinotropic action make GLP-1 an appealing candidate for the treatment of obesity and diabetes. The pharmacological value of native GLP-1, however, is limited by a short half-life [182,185,876] and dose-limited adverse gastrointestinal effects [734,877]. A common approach to enhance the therapeutic benefits of GLP-1-based pharmacology is therefore to improve its pharmacokinetics. Biochemical modification of the native peptide has enhanced exposure and time of action to improve the metabolic efficacy at tolerable doses. Furthermore, these optimized molecules allow for less frequent administration, which places less burden on the patient and improved compliance.

20.1. Optimized GLP-1 monoagonists

Several structurally refined GLP-1 derivatives with improved bioavailability and sustained action have been developed for the treatment of T2DM [878]. These drugs include exenatide (Byetta®, short-acting; and Bydureon®, long-acting, AstraZeneca), lixisenatide (Lyxumia®, Sanofi), liraglutide (Victoza®; Novo Nordisk), dulaglutide (Trulicity®; Eli Lilly & Co), albiglutide (Tanzeum®, GlaskoSmithKline), and semaglutide (Ozempic®, Novo Nordisk) [878].

Exenatide (Byetta® and Bydureon®, AstraZeneca), is a synthetic 39-amino acid peptide (first discovered as exendin-4 in the saliva of the gila monster, *Heloderma suspectum*) (Figure 8) [879]. The first 30 amino residues of exendin-4 have ~53% sequence homology to mammalian GLP-1, whereas the C-terminal nonapeptide extension has no similarities. Unlike GLP-1, exendin-4 has a glycine at the second amino acid position at the N-terminus, which protects the peptide from DPP-4-mediated degradation and inactivation. In addition, exendin-4 differs from mammalian GLP-1 by a series of amino acids in the central and C-terminal domains that include Leu10, Lys12, Gln13, Met14, Glu16, Glu17, Tyr19, Arg20, Leu21, Glu24, Lys27, Asn28, and Gly30. The C-terminal end of exendin-4 is extended relative to GLP-1 by 9 amino acids. The C-terminal extension supports a secondary structure by formation of a tryptophan cage and improves potency at the GLP-1 receptor [880]. When administered either i.v., i.p., or s.c. in rats, the half-life of exendin-4 is 18–41 min, 125–174 min, and 90–216 min, respectively [194]. Relative to GLP-1 (7–36 amide), the bioavailability of exendin-4 is nearly doubled from 36 to 67% for GLP-1 to 74–76% for exendin-4, with decelerated plasma clearance from 35 to 38 ml/min to 4–8 ml/min [194]. These pharmacokinetic parameters support twice-daily administration in humans. In patients with T2DM, exenatide decreases fasting and post-prandial levels of blood glucose [881–884], slows gastric emptying [885], reduces cardiovascular risk factors [886] and decreases body weight accompanied by suppression of food intake [885–887]. The placebo-subtracted weight loss from baseline induced by exenatide is typically in the range of low single-digit percentages. After 82 wk of treatment of T2DM patients with exenatide, the placebo-subtracted weight loss from baseline is approximately 5% [886]. A once-weekly formulation of exenatide has been developed by Amylin Pharmaceuticals and has been marketed by AstraZeneca since 2012 under the trade name Bydureon®. The primary ingredient in the formulation that allows for extended-release is polyD,L-lactide-co-glycolic acid. Together with the other excipients of the formulation, the pharmacokinetics of exenatide are improved by a mechanism involving the formation of a depot of microspheres at the injection site, which slows dissipation from the injection site [888]. Compared to classical exenatide given twice daily (BID, the once-weekly (QW) formulation at a 2 mg dose level provides a relatively stable plasma concentration at steady-state (~300 pg/ml) because of the more continuous delivery of the drug, with equal (or
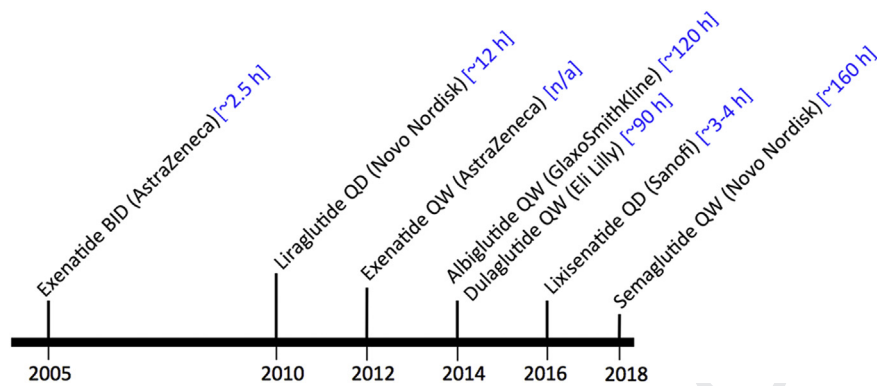
Figure 8: Timeline of GLP-1R agonists approved by the FDA for the treatment of diabetes. Numbers in parenthesis reflect the half-life of the molecules. For further explanations, please see text.

slightly improved) efficacy on glycemic control and body weight after 24 and 30 wk of treatment [888].

Lixisenatide (Adlyxin®, Sanofi) (Figure 8) is an analogue of exenatide in which the proline at position 38 is omitted and six sequential lysine residues are added to the C-terminus [889]. Lixisenatide has comparable pharmacokinetic properties relative to exenatide and is, with a half-life of ∼3–4 h, marketed for daily use in humans [890,891]. In patients with T2DM, the reduction in HbA1c is not overtly different between twice daily or once daily administration of lixisenatide [892]. Liraglutide at doses of 1.2 and 1.8 mg (Victoza®, Novo Nordisk) was approved for the treatment of type 2 diabetes in 2009 (Figure 8). Liraglutide is based on the native GLP-1 (7–37) sequence with a (conservative) substitution of lysine at position 28 with arginine. The alanine at the second position from the N-terminus is preserved in liraglutide. However, the lysine at position 20 is linked via a gamma-glutamic acid spacer to palmitic acid (C16:0), which via binding to albumin renders the drug less susceptible to DPP-4 proteolysis. The acylation also promotes multimeric formation, which decelerates diffusion from the site of injection [893]. In effect, the non-covalent binding of the fatty acid to albumin in effect prevents renal clearance. Altogether, these chemical modifications manifest in improved bioavailability and a half-life of ∼12 h [894], supporting once-daily injection in humans. Higher dose liraglutide (3 mg) has been approved for the treatment of obesity since 2014 (Saxenda®). The weight loss induced by liraglutide at 3 mg is typically in the range of ∼5–10% after 52 wk of treatment of non-diabetic obese patients [895].

Semaglutide at doses of 0.5 and 1.0 mg once weekly (Ozempic®, Novo Nordisk) has been approved for the treatment of T2DM and has been marketed since 2018 (Figure 8). Semaglutide is essentially an analogue of liraglutide. The DPP-4-sensitive alanine at the second N-terminal position in liraglutide is exchanged with an aminoisobuturic acid (Aib). Additionally, the palmitic (C16:0) fatty monoacid in liraglutide is exchanged to dicarboxylic-stearic acid (C18:0), and the link to this diacid-based fatty acid is extended relative to that in liraglutide with a synthetic spacer repeat [896]. These chemical modifications extend the half-life of semaglutide to 160 h upon subcutaneous injection in humans, supporting once-weekly administration [897]. Once-weekly injections of semaglutide dose-dependently induces body weight loss of up to 7% and reduces HbA1c by 1.8% after 40 wk of treatment in T2DM patients [898]. Semaglutide at a higher dose is being explored clinically for the treatment of non-diabetic obese patients, and an alternative oral formulation of semaglutide has

completed Phase 3 clinical trials for type 2 diabetes. In patients with type 2 diabetes, semaglutide also reduces the risk for an adverse cardiovascular event, with decreased rates of MACE (Major Adverse Cardiovascular Events, comprising cardiovascular death, nonfatal myocardial infarction and stroke) [899].

Dulaglutide at 0.75 and 1.5 mg (Trulicity®, Eli Lilly & Co) has been approved for the treatment of T2DM patients. Dulaglutide consists of two GLP-1 molecules fused at their C-termini to a human Fc fragment of immunoglobulin G4 (IgG4) via a glycine and serine-based spacer. In each of the two GLP-1 motifs, certain exendin-4 residues are introduced to replace the native GLP-1 residues. A glycine at the second N-terminal position protects the molecule from DPP-4 inactivation while a glutamic acid at position 16 stabilizes the secondary structure and improves potency. A glycine substitution at position 30, along with the native glycine at C-terminus of GLP-1 (7–37), serves as a lead–in sequence to the spacer that anchors the IgG4 Fc fragments. Together, these modifications improve bioavailability and decelerate renal clearance [900]. The resulting half-life of 90 h in humans renders the drug suitable for once weekly administration by injection (Figure 8) [901]. After 26 wk of once-weekly treatment of T2DM patients, dulaglutide at 1.5 mg reduces body weight by nearly 3% from baseline and lowered HbA1c by 1.4% [902].

Albiglutide is a head-to-tail tandem of two GLP-1 molecules in which the C-terminus of the first molecule is fused to the N-terminus of the second. Each of the two GLP-1 molecules has a glycine substitution at the DPP-4 sensitive moieties. The C-terminus of the second GLP-1 is covalently fused to human albumin, thereby slowing renal clearance and augmenting the half-life to ∼120 h in humans [903], supporting once-weekly administration to humans. Albiglutide at 50 mg (Tanzeum®, GlaskoSmithKline) is approved for the treatment of type 2 diabetes but is no longer marketed.

A common feature of the GLP-1 analogs is the dose-dependent appearance of gastrointestinal adverse effects, most commonly nausea, vomiting, and diarrhea. Compared to short-acting GLP-1 analogs (such as exenatide bidaily), long-acting analogs are associated with less nausea and vomiting but with a greater prevalence of diarrhea [734]

20.2. GLP-1-based combination therapies for individual dosing

Chemical modification of native GLP-1 has been essential to advance the pharmacological value of GLP-1R agonism for the treatment of type 2 diabetes and obesity. Nonetheless, the use of these improved GLP-1R monoagonists to treat obesity is still limited, perhaps related to the

level of body weight-lowering efficacy achieved with GLP-1R agonism alone. Higher doses of GLP-1R agonists can achieve clinically relevant weight loss, and greater increases in dose levels, in theory, can drive more weight-lowering efficacy. However, adverse gastrointestinal effects generally preclude the use of higher doses, even with adjusted uptitration dosing algorithms [734,877].

Despite the appreciable weight lowering effect of semaglutide, single use of GLP-1R monoagonism may still have limited ability to drive body weight loss into the ∼20% range, GLP-1R agonism is a vital ingredient that can be used in adjunct with other body weight-lowering hormones to optimize weight loss at tolerable doses. It seems intuitive to assume that adjunct administration of several independent hormones, each given at a tolerable dose, would enhance metabolic outcome beyond what can be achieved by each hormone alone. In an ideal case, such co-administration would engage complementary signaling mechanisms such that the magnitude of metabolic benefits might be even greater than the sum of the individual monotherapies — i.e., producing synergy. Historic examples of such a co-administration therapy for the treatment of obesity include the famous rainbow pills [878]. These pills, which enjoyed great popularity in the 1940's and 1950's, comprised a series of weight lowering substances such as thyroid hormone, amphetamines, and laxatives. However, adverse effects on the cardiovascular and brain reward systems led to their discontinued use [878]. A more recent and controlled example of a polypharmacological anti-obesity pharmacotherapy is Qsymia® (Vivus Inc.), a combination of phentermine and topiramate. Qsymia, an extended-release formulation of this combination, was approved by the FDA for the treatment of obesity in 2012. Placebo-subtracted weight loss achieved by Qsymia is typically in the range of 5—10% after up to 56 wk of treatment [904,905]. The combination of GLP-1R analogs with basal insulin has yielded promising effects to accelerate metabolic and glycemic outcomes in patients with type-2 diabetes [906—908]. Relative to the monotherapies, the benefits of this combination therapy include a greater decrease of HbA1c with an increased likelihood to achieve an HbA1c ≤ 7% [907]. Similar findings are reported for the combination of insulin with DPP-4 inhibitors [909]. Treatment of type-2 diabetic patients with a combination of the DPP-4 inhibitor sitagliptin and liraglutide showed no greater efficacy on glucose control relative to treatment with liraglutide alone, potentially because of GLP-1R saturation by the liraglutide monotherapy [910]. One study in type-2 diabetic patients reported improved glycemic control when exenatide was added to ongoing treatment with sitagliptin as compared to switching from sitagliptin to exenatide monotherapy [911].

Preclinical studies have evaluated the therapeutic potential of GLP-1 based polypharmacology for the treatment of obesity and diabetes. The various approaches include the combination of GLP-1R agonists with leptin [912,913], salmon calcitonin [914], PYY [915], CCK [916], insulin [917], adrenomedullin [918], and β3-adrenergic receptor agonists [919], and antagonists at the cannabinoid receptor 1 (CB1) [920], or agonists at the receptors for melanocortin-4 (MC4R) [685] or farnesoid-x (FXR) [921]. In summary, a series of GLP-1-based combination therapies has been found to offer metabolic benefits greater than what is achieved by treatment with each compound alone.

20.3. Unimolecular GLP-1-based polypharmacology

Another more recently developed concept of polypharmacology is to combine some of the sequences of several structurally related hormones into a single entity of enhanced potency and sustained action. The concept underlying these unimolecular multi-agonists is that a single molecule with complementary signaling through more than one receptor, each of which offers beneficial effects on systems metabolism, would maximize metabolic outcomes at tolerable doses. This strategy has refined the toolbox of weight loss pharmacology, and several molecules with agonism at key metabolic receptors are, as reviewed below, currently in clinical evaluation. One might ask why a unimolecular multi-agonist is believed to be superior over the adjunct administration of the independent hormones? Apart from the possibility that acquiring regulatory approval is seemingly easier for a single molecule relative to a co-mixture of hormones, it must be considered that each molecule in a co-mixture has its own unique pharmacokinetic profile. Simultaneously injected hormones might differ in their half-life and time of action due to differences in rates of absorption, distribution, metabolism, and clearance, as well as the potential for drug—drug interaction. While these differences might compromise the optimal metabolic outcome of a co-mixture of separate molecule entities, a single molecule might bypass some of these limitations. With a single molecule approach, the ratio of activities of the constituents, however, will be fixed, whereas individual combination therapies present the potential benefit of being titratable to allow more optimal ratio between doses of the agonists. This is particularly important when one of the components has a narrow therapeutic window.

20.3.1. GLP-1 dual- and triple-agonists

The possibility of using glucagon as a constituent of a multi-functional single molecule for the treatment of chronic metabolic diseases was initially counterintuitive due to its established role in raising blood glucose as part of the counter-regulatory physiological response to defend against insulin-induced hypoglycemia. However, the catabolic qualities of glucagon, notably its effect to decrease hepatic fat and circulating lipids, could be leveraged for therapeutic benefit in metabolic diseases if its diabetogenic action were mitigated. The prospect emerged of using GLP-1 to buffer the hyperglycemic effect of glucagon while providing complementary mechanisms to lower body weight, most notably the effects of GLP-1 to suppress appetite to coincide with the energy expenditure effect of glucagon. Co-infusion of GLP-1 and glucagon to human subjects resulted in decreased food intake, likely secondary to GLP-1 action, and increased energy expenditure, suggested to be driven by glucagon action [783,785]. Chronic co-administration of selective mono-agonists to obese non-human primates resulted in significantly more body weight loss when compared to the individual treatments, thus demonstrating the translation of these acute effects observed in humans and the chronic effects observed in primates [673].

The structural similarity of GLP-1 and glucagon as well as of their respective receptors, along with the inherent low-potency promiscuity of these hormones at the other respective receptors (apart from the pancreas, where glucagon has recently been demonstrated to also act on GLP-1R [173]), suggested the possibility of integrating both activities into a sequence-intermixed single molecule hybrid. Preclinical proof of concept of enhanced body weight lowering relative to GLP-1 mono-therapy without impaired glycemic control was first demonstrated by two independent research efforts. Both of these GLP-1/glucagon co-agonists, one based on a glucagon-derived backbone [922] and the other based on an oxyntomodulin-derived sequence [923], had superior weight loss in obese rodents relative to matched analogs that were selective in activity towards GLP-1R. Since these initial reports, other medicinal chemistry strategies have been employed to design unique GLP-1/glucagon co-agonists, including the use of exendin-4 derived sequences [924], resulting in a rich pipeline of candidates that are advancing through clinical trials [925]. Two unique compounds have been evaluated in Phase 2 trials (SAR425899 and MEDI0382), and although dual-agonism has produced promising

Review

results on body weight and glycemic control in diabetic patients [926,927], it is not apparent that these co-agonists are superior to selective GLP-1R agonists.

The original rationale for combining GLP-1 and GIP, the other predominant incretin hormone, was to leverage the individual islet effects of each hormone to impart superior glycemic control. Co-infusion of GLP-1 and GIP to healthy human subjects has resulted in increased insulinotropic effects [928].

However, controversy still exists as to whether it is best to therapeutically promote or inhibit GIP action for beneficial effects on glycemic control and body weight [929—931] as both GIPR agonists [932] and antagonists [933,934] have decreased body weight in preclinical studies as individual treatments, but also to induce synergistic body weight loss when combined with GLP-1R agonists. It is important to note that recent independent confirmations on the prospects of GIPR agonism have been demonstrated with other GIP analogs [935,936]. As with glucagon, the structural similarity of GLP-1 and GIP provided the opportunity for rationally engineering single molecules of chimeric design [937]. The first reported GLP-1/GIP co-agonists, which had prolonged half-lives due to the use of fatty acylation or PEGylation, yielded synergistic effects, lowering body weight in obese rodents relative to the individual mono-agonist comparators [465] and having a greater insulinotropic effect in non-human primates. Tirzepatide (LY3298176), a once-weekly GLP-1/GIP co-agonist, was superior to a GLP-1R agonist (dulaglutide) in preclinical studies in terms of body weight loss and glycemic control improvement [938]. In the phase 2 study of type-2 diabetic patients, Tirzepatide produced a profound reduction in body weight and improved HbA1c levels, both of which were superior relative to effects of Dulaglutide in the active comparator arm [939], thus demonstrating the translation of the preclinical observations. Whether GIP-based co-agonists can provide greater maximal clinical efficacy, relative to optimized GLP-1R agonists remains unknown, and may be further evaluated in Phase 3 studies.

Based on the encouraging efficacy of GLP-1/glucagon and GLP-1/GIP co-agonists, it was rationalized that a single molecule with triple agonism at all three of these receptors might provide even greater efficacy than the respective co-agonists. The glucagon component would presumably confer energy expenditure benefits that are noticeably absent from GLP-1 and GIP pharmacology [465,932,938], and thus permit greater body weight lowering potency and efficacy. The GIP component is hypothesized to provide an additional mechanism to buffer against the hyperglycemic effects of glucagon [940], thus permitting triple agonists of higher potency at the glucagon receptor that in theory could drive more body weight loss. Indeed, a monomeric peptide with potent and balanced triple agonism of GLP-1R, GIPR, and GcgR induced greater body weight loss than the respective co-agonists and mono-agonists in obese mice [466]. Similar preclinical benefits of a triple-acting GLP-1R, GIPR, and GCGR peptide have been observed in high-fat fed mice [941]. Readouts from the first clinical trials with triagonists will determine if such triple agonism has the curative potential that the preclinical data suggest.

20.3.2. GLP-1-based peptide-nuclear hormone conjugates

An alternative molecular format to achieve sequence-intermixed chimeric or hybridized peptides is single-molecule fusions or conjugates. As noted, the sequence similarity within the glucagon superfamily of peptides and the structural similarity within the secretin receptor family served as the molecular basis for engineering chimeric peptides with agonism at multiple receptors and with sizes comparable to the native peptides. Thus, engineering GLP-1-based multi-agonists that are hybridized sequences is likely restricted to similarly structured

hormones. However, the theoretical combinations with GLP-1 can be extended to structurally diverse hormones because multi-valent chemical fusions or conjugates are possible where the second effector molecule can vary from small oligonucleotides and nuclear hormones to peptides and large proteins.

Single molecule fusions of GLP-1 to the peptide hormones gastrin, amylin, and cholecystokinin have been reported, as have fusions of GLP-1 to larger proteins such as FGF21, and to inhibiting antibodies against PCSK9. GLP-1/gastrin fusions hold potential to re-granulate diseased islets and improve islet cell health in rodents genetically prone to develop diabetes [942]. Interestingly, modified GLP-1/gastrin fusion molecules that also incorporate bioactivity of the related gut hormone xenin have been found to have considerable preclinical efficacy [943,944]. GLP-1/amylin fusions have potential in obese diabetic patients by capitalizing on two independent pharmacological functions to reduce food intake and lower blood glucose. Pre-clinical studies with GLP-1/amylin fusions have demonstrated body weight lowering efficacy in obese rodents [945]. Similarly, GLP-1/CCK fusions can capitalize on non-redundant food intake mechanisms to lower body weight. An optimized GLP-1/CCK fusion had enhanced efficacy to lower body weight in obese rodents relative to mono-agonist controls and their co-administration [946,947]. FGF21 analogues have been observed to potently lower triglycerides in T2DM patients [948] and to promote energy expenditure in rodents. Thus, GLP-1/FGF21 fusions have the potential, at least in rodents, to provide greater improvements in dyslipidemia than GLP-1R agonists and have the potential to drive greater body weight loss through the energy expenditure mechanism not evident with GLP-1 mono-therapy. GLP-1/FGF21 fusions have been reported to dose-dependently lower body weight in obese mice, and co-administration of the respective mono-agonists results in synergistic body weight loss in obese mice (abstracts). The combination of GLP-1 and anti-PCSK9 antibodies, like the GLP-1/FGF21 combinations, has great promise in diabetic patients at risk of cardiovascular disease due to effects on hypercholesterolemia. A pharmaceutically-optimized GLP-1/anti-PCSK9 antibody was reported to have potent weight lowering efficacy in obese rodents and to have potent effects to lower cholesterol in cynomolgous monkeys [949]. In a recent phase I clinical study, treatment of overweight and obese patients with GLP-1/anti-PCSK9 decreased LDL cholesterol but failed to improve glucose metabolism [950].

Oncolytic drug discovery research has been at the forefront of tissue-targeting strategies as a pharmacological tool to improve the efficacy of immunological agents by empowering them with classical chemotherapeutic mechanisms, a procedure which also results in an improved therapeutic window for the classical small molecule. By leveraging the high specificity of the antibodies and employing meta-stable covalent linkages of a cytotoxic payload, this effector molecule is targeted specifically to the antigen-producing cells. Once targeted, the cytotoxic agent is channeled into the cell via the antibody internalization process where it is released internally to provide a cytotoxic mechanism that is complementary to the immunological effects of the antibody. A similar concept has emerged in metabolic disease drug discovery research, although it is fundamentally different from the oncology field, because the effector molecule is not meant to drive a cell-killing effect but a cell-altering effect.

GLP-1R analogs have been leveraged for their relatively selective modalities of action in pancreatic islets and the central nervous system to precisely deliver cargo such as antisense oligonucleotides and nuclear hormones to these tissues. The aim is to alter the cellular physiology of these specific cell subtypes by the action of the effector molecule while simultaneously capitalizing on the therapeutic benefits of the GLP-1 targeting molecule. GLP-1/antisense conjugates have

been developed to bypass a refractory nature of pancreatic β-cells to the uptake of antisense oligonucleotides while harnessing the untapped potential of these oligonucleotides to repress the expression of pathological intracellular targets [951]. Conjugates of particular anti-sense oligonucleotides to a GLP-1 analog enhanced the productive uptake of the oligonucleotide in GLP-1R expressing cells to elicit selective and effective gene silencing *in vitro* and *in vivo* with limited off-target accumulation of the oligonucleotide. Although the therapeutic benefits have yet to be reported for a specific GLP-1/antisense molecule designed to deliver a therapeutically-relevant antisense, the data suggest that GLP-1R is a suitable vector for *in vivo* tissue targeting, cellular internalization, and intracellular deposition of a cargo moiety. However, a comprehensive application of GLP-1R mediated targeting has yet to be demonstrated though translational pharmacology experiments.

In other pre-clinical experiments, GLP-1 analogs have been utilized to selectively deliver nuclear hormone payloads to cells in a targeted manner. Estrogen has pleotropic, beneficial effects on metabolism yet the reproductive endocrine toxicity and tumorigenic potential limits its use in chronic settings other than hormone replacement therapy. Emerging preclinical data suggest that a GLP-1/estrogen conjugate enhances the anorectic, body weight lowering, insulinotropic, and islet preservation effects in mice relative to the GLP-1 analog or estrogen alone [467,952,953]. Importantly, this GLP-1/estrogen conjugate was devoid of the uterotrophic effect evident with estrogen alone, demonstrating an improved therapeutic index mediated by the GLP-1 component. GLP-1R targeting has also been leveraged to deliver the anti-inflammatory properties of dexamethasone to metabolically relevant tissues. Although glucocorticoids are potent anti-inflammatory molecules, chronic treatment oftentimes results in compromised metabolic control, notably due to hepatic action to increase glucose production. In mice, a GLP-1/dexamethasone conjugate circumvented action in the liver and skeletal system to avoid the deleterious effects on glycemic control and bone turnover, respectively, that is evident with systemic dexamethasone action [820]. This GLP-1/dexamethasone conjugate improved the body weight lowering potency of the GLP-1 analog alone, partially by alleviating surrogate markers of hypothalamic inflammation. Despite these provocative preclinical findings suggesting a targeting phenomenon that results in enhanced efficacy and improved therapeutic index for these nuclear hormone conjugates, more studies are required to delineate the mechanism for the intracellular delivery and release of the cargo along with precise pharmacokinetic analyses. Furthermore, this targeting principle requires additional validation, particularly in the context of human biology, before tangible conclusions regarding its applicability to clinical pharmacology can be made.

21. OUTLOOK

Depending on the molecule (native or recombinant long-acting) and the route of administration, GLP-1 analogs have broad pleiotropic action on metabolism. Among the numerous beneficial effects mediated by GLP-1R agonists are regulation of blood glucose, lowering of body weight via inhibition of food intake and the decrement of gastric motility, stimulation of cell proliferation, reduction of inflammation and apoptosis, improvement of cardiovascular function, and neuro-protection. Recombinant GLP-1R analogs with improved pharmacokinetics and sustained action are successful in the treatment of type-2 diabetes, and there is considerable and ongoing effort to further optimize their action profile to improve therapeutic outcome and patient compliance. Novel peptides that combine the pharmacology of GLP-1 with that of other gut peptides are in clinical evaluation for the treatment of diabetes and obesity. While long-term clinical studies are ongoing, we can be carefully optimistic that improved GLP-1-based pharmacology can one day be safely applied to further lower body weight relative to currently available GLP-1R agonists.

CONFLICT OF INTEREST

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MOLECULAR
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MOLECULAR
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MOLECULAR METABOLISM

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