GASTROINTESTINAL HORMONES

EDITED BY: Silvano Paternoster, Damien Keating and Marco Falasca
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GASTROINTESTINAL HORMONES

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EDITORIAL
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Editorial: Gastrointestinal Hormones

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Keywords: glucagon-like peptides, gastrointestinal hormone, G protein coupled receptor (GPCR), NAFLD, enteroendocrine cell

Editorial on the Research Topic

Gastrointestinal Hormones

The gastrointestinal tract is a vast organ hosting a broad gamut of hormone secreting cells. After more than a century since the first description in 1906 of metabolically active gut extracts by Moore (1), we are still unveiling novel functions in health and disease for this complex endocrine organ.

In the current Research Topic, we picture our current understanding of the enteroendocrine cell system (EECs), with new layers of complexity, covering not only the physiology and pathophysiology of the most well-characterized gut peptides Glucagon-like peptide-1 (GLP-1) and the Gastric insulinotropic peptide (GIP), but also many other mediators of central metabolism.

In this issues Sun et al. discuss the most recently recognized physiological impact of different gut hormones including GIP, GLP-1, Oxyntomodulin, peptide YY (PYY), serotonin, ghrelin, and the less studied insulin-like peptide 5 (INSL-5). The first four, known primarily for their anorexigenic, satiety-inducing action, while the last two for their orexigenic, appetite-inducing activity, orchestrate our metabolic response to food, maintaining our glucose, and energy homeostasis. The role of serotonin (5-HT) is also highlighted by Beyder, as a key mediator of gut-mechanosensation.

Recent studies have expanded our understanding of the physiology of different gut hormones, namely GLP-1, GLP-2, GIP, and PYY in bone metabolism. Schiellerup et al. discusses the most recent evidence updating us on the potential development of drugs based on these peptides for the management of diseases affecting bone metabolism, such as osteoporosis.

In two other reviews, an updated picture of the gut chemo-sensation of sweet and bitter tastants is dissected. Kreuch et al. delves deeper into the most recent findings surrounding the ability to detect dietary sugars, considering the recent rise in ingested non-caloric sweeteners and their impact on enteroendocrine cells and the microbiome, ultimately affecting satiety and glycaemia. Influence of this complex gut-brain axis holds a yet untapped potential for the management of chronic metabolic diseases such as obesity and diabetes. Similarly, understanding of the bitter sensation in the gut, highlighted by Xie et al., has important clinical implications. The chemo-sensation of bitter molecules beyond the tongue is transduced by different G-protein coupled receptors, namely different species of type 2 receptors (T2Rs) expressed by the enteroendocrine cells. This powerful gut axis is another tool with therapeutical implications for the modulation of different gut hormones, including the anorexigenic GLP-1, PYY, cholecystokinin (CCK), and the orexigenic Ghrelin.

Nonetheless, among all the gut-derived hormones, GLP-1 is the only one, which biology is currently exploited for the treatment of metabolic pathologies such as type 2 diabetes and obesity. Rowlands et al. expand upon the implications of GLP-1 mimicking drugs, consider the molecular targets in both acute and chronic settings, and dissect the tissue-specific downstream signaling pathways behind the recognized cardiometabolic benefits of these therapeutics.

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Drugs activating the GLP-1 receptor are also beneficial in the management of another current epidemic, namely non-alcoholic fatty liver disease (NAFLD). Seghieri et al. delves deeper into this topic by reviewing the clinical benefits of molecules designed to activate both GLP-1 and Glucagon receptors, with superior anti-NAFLD properties. Nevertheless, the physiology and pathophysiology of GLP-1 is indeed much more complex than initially thought, with GLP-1 now thought to not only be acting systemically as a hormone through the bloodstream. Our understanding of gut-derived, and pancreas derived GLP-1, are both analyzed and repurposed by Paternoster and Falasca drawing more attention on the tissue-specific action of different GLP-1 species, including the once thought inactive, and much more abundant, metabolites.

Rehfeld encourages us to broaden the old concept of gut hormones inducing the release of pancreatic hormones, or incretins, currently epitomized mainly by GLP-1 and GIP. The currently recognized physiological axis linking the gut to the pancreas for whole-body metabolic control sees hundreds of different peptides, often with overlapping functions and possibly synergistic actions of yet unknown biology and metabolic impact.

A better understanding of the physiology of EECs and their involvement in a variety of physiological pathways and pathophysiological phenomenon will likely provide a strong platform for future therapeutics designed to address the morbidity of chronic metabolic diseases such as obesity and type 2 diabetes.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Gut Hormones and Their Effect on Bone Metabolism. Potential Drug Therapies in Future Osteoporosis Treatment

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Bone homeostasis displays a circadian rhythm with increased resorption during the night time as compared to day time, a difference that seems—at least partly—to be caused by food intake during the day. Thus, ingestion of a meal results in a decrease in bone resorption, but people suffering from short bowel syndrome lack this response. Gut hormones, released in response to a meal, contribute to this link between the gut and bone metabolism. The responsible hormones appear to include glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), known as incretin hormones due to their role in regulating glucose homeostasis by enhancing insulin release in response to food intake. They interact with their cognate receptors (GIPR and GLP-1R), which are both members of the class B G protein-coupled receptors (GPCRs), and already recognized as targets for treatment of metabolic diseases, such as type 2 diabetes mellitus (T2DM) and obesity. Glucagon-like peptide-2 (GLP-2), secreted concomitantly with GLP-1, acting via another class B receptor (GLP-2R), is also part of this gut-bone axis. Several studies, including human studies, have indicated that these three hormones inhibit bone resorption and, moreover, that GIP increases bone formation. Another hormone, peptide YY (PYY), is also secreted from the enteroendocrine L-cells (together with GLP-1 and GLP-2), and acts mainly via interaction with the class A GPCR NPY-R2. PYY is best known for its effect on appetite regulation, but recent studies have also shown an effect of PYY on bone metabolism. The aim of this review is to summarize the current knowledge of the actions of GIP, GLP-1, GLP-2, and PYY on bone metabolism, and to discuss future therapies targeting these receptors for the treatment of osteoporosis.

Keywords: gut hormones, bone metabolism, GIP, GLP-1, GLP-2, PYY, osteoporosis

INTRODUCTION—BONES

Bone is a tissue with very important mechanical functions, providing rigidity, strength and shape, and is essential for movement. However, in spite of its apparently static structure, bone tissue is dynamic and undergoes a constant remodeling, consisting of processes of bone resorption and bone formation. Proper balance is controlled by the coupling of these two processes, and involves a number of coordinated signaling mechanisms. In normal bone remodeling, a balance between bone resorption (mediated by osteoclasts) and bone formation (mediated by osteoblasts) is maintained to ensure a constant bone mass. An imbalance between bone resorption and bone formation may occur under certain pathological conditions, and lead to abnormal bone remodeling and the development of bone disorders (1). Bone also has an important function as a reservoir for calcium and phosphate, bound in the matrix as hydroxyapatite, and bone tissue is, therefore, along with the intestine and kidneys, important for the maintenance of proper calcium levels (2–4).

Histologically, there are two main types of bone, cortical and trabecular. These have different structure and properties. Cortical bone has a highly organized, lamellar structure, providing planar strength. Generally, bones have an outer layer of cortical bone with trabecular bone beneath. The weight-bearing long bones, such as the femur and humerus, mainly have cortical bone in their shafts. Trabecular bone has a more irregular and less dense structure, consisting of interconnecting bars, or trabeculae, with bone marrow filling the gaps. The number of trabeculae has been shown to be more important than their thickness in regard to the strength of the bone (5).

Bone tissue consists of cellular elements within an extracellular matrix. The cellular components are the osteoblasts, the osteocytes and the osteoclasts. The bone-forming osteoblasts derive from mesenchymal stem cells that differentiate into osteoprogenitor cells, a process that is dependent on the Wnt/β-catenin pathway. With age, the osteoblasts become buried in the matrix and are now designated osteocytes. These cells communicate with each other and with other cells, particularly those on the surface of the bone tissue, through dendritic processes in canaliculi in the bone, allowing them to regulate bone-turnover in response to mechanical stress (1). Osteoclasts are multinucleated cells, derived from the macrophage/monocyte lineage, which resorb bone on the growth surface. The differentiation into mature osteoclasts is dependent on activation by the receptor activator of nuclear factor κB (NF-κB) ligand (RANKL) and monocyte colony stimulating factor (M-CSF) produced by the osteoblasts.

The remodeling of the bone involves a coordinated action of a team of cells, referred to as a basic multicellular unit (BMU). The osteoclast-mediated resorption and the osteoblast-mediated formation are thought to be orchestrated by local regulation within the BMU, as well as by systemic regulation from outside the BMU. The local signaling within the BMU is often presented as a complex network of regulation between the different cell types, where the osteocyte population regulates the activity of the osteoblast population, and the osteoblast population, in turn, regulates the activity of the osteoclast population and vice versa (**Figure 1**). Osteocytes regulate the osteoblasts via signaling molecules including fibroblast growth factor-23 (FGF-23), bone morphogenetic proteins (BMPs), and sclerostin, whereas some of the key signaling molecules involved in the osteoblastic regulation of the osteoclasts include RANKL, that induces osteoclast activity, and osteoprotegerin (OPG), that counters the effect of RANKL. Malfunctions in some of these regulatory mechanisms are known to cause bone disorders; mutations of FGF-23, for example, are known to cause autosomal dominant hypophosphatemic rickets (ADHR). Some of these regulatory factors, including RANKL and sclerostin, are targets for therapies that aim to alter the balance between the osteoblast activity and osteoclast activity in order to treat osteoporosis, which is characterized by an imbalance between these activities.

When bone is resorbed, a carboxy-terminal telopeptide of type I collagen (CTX) is liberated and released into the blood stream. Levels of circulating CTX are, therefore, used as a biomarker for bone resorption. CTX levels show diurnal variation, peaking during the night-time, and showing a nadir in the late afternoon. As fasting is associated with a flattened circadian rhythm (6), with the day-time decline in CTX levels being eliminated, ingestion of food seems to be the explanation for the daytime suppression (7, 8). Bone formation can be assessed by measurements of an amino-terminal propeptide of type 1 procollagen (P1NP) or by measuring the protein osteocalcin, which is secreted from osteoblasts.

THE GUT-BONE AXIS

The gut and the bones are connected through the gut-bone axis, and this interaction is mediated by hormones secreted from the intestine. These hormones are secreted in response to food intake and causes a decrease in bone resorption (7–9), and are thus mediators of the adaptation of bones to nutrient availability. Bone resorption is increased during the night as compared to the day, and this day-time suppression is eliminated by fasting, further affirming the role of the gastrointestinal hormones in the control of bone homeostasis. Many hormones are secreted from the gut [for a recent review see Gribble and Reimann (10)], but the focus of this review will be on the incretin hormones glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), as well as glucagon-like peptide-2 (GLP-2) and peptide YY (PYY).

GIP (secreted from enteroendocrine K-cells) and GLP-1 (secreted from L-cells) have been extensively studied with respect to their effects on glucose metabolism as mediators of the incretin effect: i.e., the enhanced insulin secretion which occurs when glucose is ingested orally compared to i.v. glucose injection (11–14). For this reason, there has been much interest in their use in the treatment of type 2 diabetes mellitus (T2DM) and obesity, and many widely used drugs for T2DM, such as liraglutide and exenatide, act as GLP-1 receptor agonists (GLP-1RA). GLP-2 is also released from the L-cells in the small intestine, but in contrast to the glucose-lowering effects of GLP-1 (and GIP), GLP-2 is mostly known as an intestinotropic factor (15, 16). GIP, GLP-1,

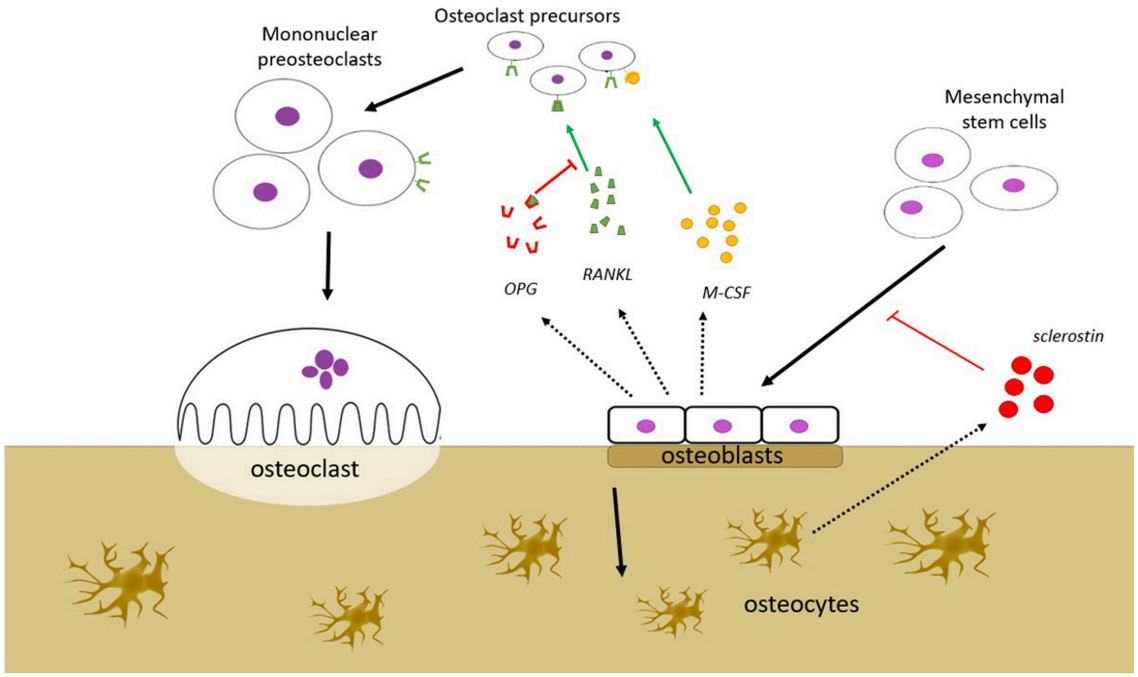

FIGURE 1 | Bone remodeling is a process that takes place in the bone remodeling unit (BMU). BMU consists of bone resorbing osteoclasts (OC), bone forming osteoblasts (OB), and osteocytes. The process is regulated by local signals between the cells and by external stimuli. OBs stimulate OC precursors to differentiate into mature OCs by secreting monocyte colony stimulating factor (M-CSF) and RANKL, but may also inhibit the same cells by secreting osteoprotegerin (OPG) that scavenges RANKL, preventing it from binding to the RANK receptors on the OC precursors. OBs are derived from mesenchymal stem cells, a process that is dependent on the Wnt/β-catenin pathway. This pathway is inhibited by sclerostin, which is secreted from osteocytes. Several other factors which affect bone remodeling are mentioned in the text (FGF-23, BMP), but are not shown in this figure.

and GLP-2 are all believed to protect against bone resorption, either via direct effects on the bone cells, or indirectly. PYY is co-secreted with GLP-1 and −2 from the L-cells and also affects bone metabolism, possibly by inhibiting formation. These hormones are involved in the gut-bone axis, as reviewed below and summarized in **Table 1**.

Glucagon-Like Peptide-2 (GLP-2)

GLP-2 is co-secreted with GLP-1 from intestinal L-cells in the small and large intestine upon nutrient ingestion. GLP-1 and GLP-2 are derived from pro-glucagon which is post-translationally processed by pro-hormone convertase 1/3 in the L-cells (58–60). Intact GLP-2(1-33) (referred to as GLP-2 in rest of this review) is cleaved by the ubiquitous protease dipeptidyl peptidase-4 (DPP-4) at the alanine in position 2, with a half-life in plasma of approximately 7 min, forming the main degradation product GLP-2(3-33) (61). This variant has been shown to act as a low affinity, partial agonist with competitive antagonistic properties on the GLP-2 receptor *in vitro* (62). A prolonged half-life of GLP-2 can be achieved by substitution of the alanine in position 2 or by the use of DPP-4 inhibitors (63–65).

The GLP-2 receptor (GLP-2R) belongs to class B G protein-coupled receptors (GPCR). Based on animal studies, it is predominantly expressed in the gastrointestinal tract in enteric neurons (66, 67), but has also been found in the central nervous system and may be sparsely expressed in the lungs (20, 66). The exact localization of the GLP-2R in humans is however still uncertain due to lack of good antibodies for immuno-localization, as well as the low levels of GLP-2R expression in cells outside the gastrointestinal tract; extrapolation from other species may be risky because of differences between species (68).

GLP-2 has trophic effects on the intestine. In mice, administration of GLP-2 promotes growth of the small and large intestine, whereas co-administration of GLP-2 and high doses of GLP-2(3-33) results in a reduced response (62). GLP-2 acts on the intestinal crypt compartment, stimulating proliferation, but also inhibits apoptosis (69). Accordingly, GLP-2 has been studied in patients with short bowel syndrome (SBS) (70) and, since 2012, a DPP-4 resistant GLP-2 analog (teduglutide) has been used in the treatment of SBS. GLP-2 also appears to improve the intestinal barrier function, up-regulate glucose transport and increase mesenteric blood flow. Although less well-established, it has also been reported to inhibit food intake and promote neuronal proliferation (17, 18, 71, 72). The mechanisms underlying the effects of GLP-2 are not well-described, although they seem to be mediated indirectly through the ErbB system (73), keratinocyte growth factor (KGF) (74) and, perhaps, insulin-like growth factor-1 (IGF-1) (69).

GLP-2's Effect on Bones

In 2001, the first human study revealed that 5-weeks treatment with natural GLP-2 significantly increased spinal areal bone

TABLE 1 | Summary of the known effects of the gut hormones GLP-2, GIP, GLP-1, and PYY on bone metabolism.

Hormone	Study design	Species	Finding	References
GLP-2	*In vivo*	Human	GLP-2 inhibits bone resorption (measured as CTX) with only minimal effects on bone formation (measured as osteocalcin or P1NP). Four months of GLP-2 treatment increases hip BMD in postmenopausal women	(7, 17–19)
		Mouse	None. No studies report effects of GLP-2 on bone remodeling in the mouse	
		Rat	None	
	In vitro	Human	The GLP-2R has not been identified in human bone cells, though one study reports GLP-2R expression on the cell lines MG-63 and TE-85 (reflecting immature human osteoblasts)	(20)
		Mouse	None	
		Rat	None	
Summary GLP-2: GLP-2 does not affect bone remodeling in rodents either *In vitro* or *In vivo*. In humans, GLP-2 acutely inhibits bone resorption, and 4 months of GLP-2 treatment increases hip BMD in postmenopausal women				
GIP	*In vivo*	Human	GIP reduces CTX independently of insulin Loss-of function GIPR gene polymorphism is correlated to a decreased BMD and increased fracture risk	(9, 21–23)
		Mouse	Lack of GIP signaling or peptide alters the bone structure in a negative direction, but findings in different studies are not always consistent	(24–27)
		Rat	GIP Improves bone density in OVX rats and cortical bone properties	(28, 29)
	In vitro	Human	GIP reduces osteoclast formation and resorption In osteoblastic cell lines, GIP increase stimulates ALP and PINP, and diminishes cell death	(20, 30–32)
		Mouse	GIP inhibits PTH induced resorption and stimulates ALP and mineralization in osteoblasts	(33, 34)
		Rat	None	
Summary GIP: GIP has a direct effect on regulation on bone metabolism with anabolic effects on osteoblasts and anti-resorptive effects on osteoclasts				
GLP-1	*In vivo*	Human	GLP-1 has positive effects on bone metabolism, possibly through increased formation. No effect on plasma CTX concentrations	(7, 22, 35)
		Mouse	Studies in mice show that treatment with GLP-1RAs have protective effects against OVX-induced or diabetes-induced bone loss	(36, 37)
		Rat	GLP-1 has positive effects on bone strength and quality, and protects against bone loss. It causes an increase in bone formation parameters and a decrease in bone resorption parameters	(38–44)
	In vitro	Human	The receptor has been found on some osteoblastic cell lines. GLP-1 also increases cell viability and promotes osteogenic differentiation of BMSCs	(20, 38, 40, 45)
		Mouse	The receptor has been found in mouse osteoblast-like cells. In most studies, GLP-1 leads to an increase in differentiation and proliferation of osteoblasts, and it also exerts effects on osteoclasts	(37, 46–49)
		Rat	The GLP-1R has been found on rat osteoblasts and osteocytes, and GLP-1 affects the osteoblastic differentiation and regulate osteocyte protein production	(39, 40)
Summary GLP-1: GLP-1 directly affect bone cells, and regulates bone turnover by increasing formation and decreasing resorption				
PYY	*In vivo*	Human	Inverse relationship between plasma PYY and BMD in populations with weight gain (↓PYY and ↑BMD in obesity) and weight loss (↑PYY and ↓BMD in patients with anorexia and after gastric bypass surgery)	(50–52)
		Mouse	Direct effect of PYY on osteoblast and osteoclast activity with a negative relationship between PYY and osteoclast activity $PYY^{-/-}$ mice exhibit an increase in bone mass and strength, although controversies exist Regulation of bone resorption and formation seem to occur via Y1, Y2, and Y6 receptors	(53–57)
		Rat	None	
	In vitro	Human	None	
		Mouse	PYY signaling in osteoblasts occurs via Y1 receptors, but no Y receptors seem to exist on osteoclasts	(54)
		Rat	None	
Summary PYY: PYY may play a role in bone mass regulation as evident from association studies in populations with altered energy balance. Support of this originates from rodent studies				

mineral density (aBMD) in SBS patients with no terminal ileum and no colon (70, 75). Shortly hereafter, Henriksen et al. showed that GLP-2 administered subcutaneously (s.c.) in doses ranging from 200, 400, and 800 μg in healthy postmenopausal women dose-dependently reduced bone resorption (measured as CTX), while the bone formation (measured as osteocalcin) was unaffected (7). Moreover, GLP-2 s.c. injected at bedtime also inhibited the nocturnal bone resorption (CTX) (76). In a 14-day study, daily bedtime injections of 1.6 and 3.2 mg GLP-2 were well-tolerated and reduced CTX with no effect on markers of bone formation (osteocalcin and P1NP) (19). Finally, 4 months of GLP-2 treatment resulted in a dose-related significant increase in hip aBMD of about 1%, with no signs of GLP-2 antibodies or tachyphylaxis (17). In contrast to the earlier findings that GLP-2 increased aBMD in SBS patients (75), Gottschalck et al. reported that reductions in CTX after exogenous GLP-2 requires an intact small intestine, indicating an indirect effect of GLP-2 involving the intestine (77, 78). Additionally, they found that GLP-2 decreased PTH in control participants with an intact intestine, making PTH a potential mediator of the GLP-2 induced decrease in CTX (77). In 2013, Askov-Hansen et al. investigated the effect of high concentration (achieved by i.v. injection) vs. prolonged exposure (achieved by s.c. injection) of GLP-2 in healthy participants. They found that prolonged exposure was more effective in reducing circulating CTX levels than acute high concentrations. Pre-treatment with the DPP-4 inhibitor sitagliptin increased plasma levels of GLP-2, but had no additional effects on CTX (18). Intriguingly, despite the impact of GLP-2 on osteoclast activity, the GLP-2R has not been identified in human osteoclasts, or in any other bone-related cell type, though Pacheco-Pantoja et al. found the receptor to be expressed in the immature human osteoblast cell lines MG-63 and TE-85 (20).

In summary, GLP-2 markedly inhibits bone resorption with only minimal effects on bone formation, resulting in an increased bone mineral density. Judged from existing studies, only supra-physiological doses of exogenous GLP-2 reduce bone resorption (CTX), but the mechanism by which GLP-2 affects bone metabolism is still unknown. It might act directly on bone cells or the effect might be mediated indirectly, possibly involving other intestinal factors.

Glucose-Dependent Insulinotropic Polypeptide (GIP)

GIP is a 42 amino acid peptide secreted upon food ingestion from the enteroendocrine K-cells located primarily in the proximal small intestine (71). Together with GLP-1, it is known as an incretin hormone, being responsible for approximately 50–70% of the insulin response to oral glucose administration in healthy humans (71). GIP(1-42) is, like GLP-2, N-terminally cleaved by DPP-4 generating the metabolite GIP(3-42) (79), thereby resulting in a plasma half-life of active GIP of 4 min in humans (80). For research purposes, several DPP-4 resistant GIP analogs have been produced, such as N-AcGIP, Pro^3GIP, and D-Ala_2-GIP. Moreover, a naturally occurring C-terminally truncated variant, devoid of the last 12 amino acids, GIP(1-30)NH_2, acts as full agonist for the human GIP system (81). DPP-4 cleavage of this compound results in GIP(3-30)NH_2, a high affinity competitive antagonist for the GIP system with proven activity in humans (81–83).

The GIP receptor (GIPR) belongs to the class B GPCRs, and stimulates the $G\alpha_S$ adenylyl-cyclase-cAMP-PKA pathway. It is expressed in a wide range of tissues and organs, the most important being the endocrine pancreas, adipose tissue, bone, and several CNS regions (71). Accordingly, GIPR signaling has been demonstrated in pancreatic α- and β-cells (84), bone cells (30), adipocytes (85), and hippocampal neural cells (86).The GIP system is less conserved among species compared to the GLP-1 system (87, 88). Thus, the sequence homology of the GIPR between rodents and humans is only 81%, and the GIP peptide has 2 and 3 amino acid substitutions, respectively, in rats and mice compared to human GIP (88, 89). The efficacy of human GIP on rat and mice GIPRs is only 75 and 60%, respectively, of those of rat and mouse GIP, respectively. This information is relevant for *in vivo* and *in vitro* tests of the GIP system in different species (89).

Due to a markedly impaired insulinotropic effect of GIP in T2DM patients (putatively due to a desensitization of the GIP system) (90), the focus for GIP research has changed somewhat from pancreatic β-cell stimulation and glucose homeostasis to other areas, such as metabolism of bones and adipocytes, and neural diseases.

GIP's Effect on Bones

The GIPR is expressed in both osteoblast- and osteoclast-derived cell lines (20, 46), and in murine primary cultures of osteoclasts and osteoblasts (33, 34). Aoyama et al. found that the expression of the GIPR increased upon increasing glucose concentrations in the media (46). Expression of the GIPR has, moreover, been verified on human bone marrow-derived mesenchymal stem cells (BMSC) (31).

The osteoblastic cell lines vary in the degree of their maturity, a difference that has been suggested to correlate with GIPR expression. Moreover, the anabolic impact of GIP stimulation on bone parameters such as alkaline phosphatase (ALP), P1NP and cell viability varies between cell lines (20). GIP increases intracellular calcium $[Ca^{2+}]_i$ and cAMP, and increases expression of P1NP and ALP activity (30). It also increases the expression of c-Fos, an important factor in bone cell proliferation and differentiation (45). Furthermore, GIP improves collagen maturity and fibril diameter in a cAMP dependent manner (91), and stimulates both ALP and mineralization in primary osteoblast cultures from murine BMSC (34). Finally, GIP attenuates caspase 3/7 activity and, thereby, diminishes cell death in both hBMSC and an osteoblastic cell line (31). In primary murine osteoclast cultures, GIP inhibits PTH-induced bone resorption (33). Another study showed that GIP reduces osteoclast formation and resorption in primary human and murine cultures, and this reduced differentiation is independent of the conventional adenylyl-cyclase-cAMP-PKA pathway. GIP decreased the RANKL-induced $[Ca^{2+}]_i$ increase and calcineurin activity, and decreased nuclear translocation of the RANKL

downstream target, NFATc1, which is important for terminal osteoclast differentiation (32).

The first *in vivo* study was conducted in 2001, where Bollag et al. showed a positive impact of native GIP on bone density in ovariectomized (OVX) rats (28). Since then, many studies have been performed using DPP-4 resistant peptides and genetically modified mice with either receptor knock-out (KO), congenital overexpression or deficiency of the GIP peptide. The DPP-4 resistant peptides all show either anabolic or anti-resorptive properties. N-AcGIP, for example, improved cortical bone properties in rats and decreased osteoclast mediated bone resorption in OVX mice, as seen by a reduction in both the number of osteoclasts and the resorption marker CTX (29, 32). In a type 1 diabetes (T1DM) mouse model, short term treatment with D-Ala_2-GIP, prevented a reduction of bone formation parameters and at tissue level, it improved mechanical properties (36). A peptide hybrid of GIP and oxyntomodulin, stimulating both GIP, GLP-1, and glucagon receptors, also improved cortical bone strength in a T2DM mouse model (92).

Two variants of GIPR KO mice exist, varying in the amount of exons deleted. Both variants have compromised bone properties, but some of the results are conflicting. The first GIPR KO mouse characterized, with deletion of exon 4–5, showed decreased bone formation parameters such as aBMD, bone mineral content (BMC), trabecular bone volume, ALP and osteocalcin, and increased resorptive parameters, such as increased numbers of osteoclasts and increased urinary elimination of the resorption marker, deoxypyridinoline (24, 25). The other GIPR KO mice, with deletion of exon 1-6, showed decreased bone strength and cortical thickness, and increased bone resorption, but they also had an increased number of osteoblasts and a reduced number of osteoclasts (26, 27). The double incretin KO mouse, a combination of a GIPR KO and GLP-1R KO, showed reduction in the cortical properties and a reduced strength of the bones (93). Congenital deficiency of the GIP hormone was similarly consistent with an important role of GIP for bone metabolism, showing decreased bone volume and number of trabeculae, and increased osteoclast surfaces (94). Conversely, overexpression of GIP was associated with increased formation of bone, with an increase in bone mass, number of osteoblasts, osteocalcin levels, and inhibited bone resorption, as indicated by decreased pyridinoline crosslinks and decreased number of osteoclasts (34, 95).

In an early human study evaluating the effects of a brief intravenous injection of GIP, there were no apparent effects on bone resorption (7). A more recent study showed a 50% decrease in CTX upon oral glucose ingestion, and 30% decrease upon intravenous glucose administration, thus with a larger decrease in the presence of high levels of incretin hormones (9). Two subsequent studies showed a robust direct inhibitory effect of GIP infusion on CTX at both low, eu-, and hyperglycemic levels (21), and that the reduction of CTX by GIP was independent of insulin (22). Moreover, a loss-of function GIPR gene polymorphism (E354Q) was correlated with decreased aBMD, as analyzed by DXA-scans in a 10 year follow up study of 1424 perimenopausal women, and an evaluation of registered fractures over a period of 16 years showed a 50% increased fracture risk (23).

Overall, the studies in both humans and rodents indicate GIP to be a pivotal and direct regulator of bone metabolism, with direct anabolic effects on osteoblasts and anti-resorptive effects on osteoclasts.

Glucagon-Like Peptide 1 (GLP-1)

GLP-1 is encoded within the proglucagon gene, which also codes for glucagon and GLP-2. In the α-cells of the pancreas, the proglucagon peptide is cleaved by prohormone convertase 2 (PC2), yielding glucagon. In the intestinal L-cells, PC1/3 cleaves proglucagon to give the peptides, GLP-1 (PG78-107) (58, 59) and GLP-2. GLP-1 is found in a glycine-extended form, GLP-1 (7-37), which can be C-terminally amidated to give GLP-1 (7-36 NH_2) (59). It is released primarily in response to nutrient intake, and is less affected by endocrine and neuronal factors. Thus, there is no apparent "cephalic phase" for the meal-induced response (96). As for GIP and GLP-2, GLP-1 is degraded by the enzyme DPP-4 (97), which cleaves it after its alanine in position 2, giving it a half-life <2 min (61, 71, 97–99).

The GLP-1R is a class B GPCR, related to the GIP-, GLP-2- and glucagon receptors and like these, it mainly couples to Gαs (71). Although it is internalized, desensitization may not occur *in vivo* (100). The GLP-1R is found in a variety of tissues, amongst them, the pancreas and CNS, where it regulates release of glucoregulatory hormones and appetite, respectively. GLP-1 acts on the pancreatic β-, α-, and δ-cells, where it stimulates insulin secretion, inhibits glucagon secretion and stimulates somatostatin release, respectively. Moreover, GLP-1R activation in the CNS leads to decreased food intake and weight loss (14), while in the stomach, GLP-1 inhibits gastric motility and acid secretion (71). Due to these beneficial effects of GLP-1R activation on metabolism, several drugs have been developed that act on the GLP-1 receptors, including the GLP-1RAs liraglutide, dulaglutide and exendin-4, used in the treatment of T2DM and obesity (101).

GLP-1's Effect on Bones

Several studies indicate that GLP-1 has an effect on bone homeostasis, although the exact mechanism involved remains unclear. The GLP-1R has been found on immature osteoblastic TE-85 and MG63 cells, but not in the Saos2 cell line (20). It was also found in mouse osteoblast-like MC3T3-E1 cells (46, 47), with one of the studies showing that its expression increases with higher glucose concentrations in the media (46). In another study, a GLP-1 receptor distinct from the classical pancreatic-type receptor was found on MC3T3-E1 cells (102). In a study by Meng, the receptor was found in bone marrow stem cells (BMSC). These cells can differentiate into osteoblastic cells, but the GLP-1R has not been found in primary osteoblasts (38), or on the osteocyte-like MLO-Y4 cells (39). Pereira et al. (37) found that the GLP-1R was expressed in mouse bone and bone marrow, and in primary osteoblasts and osteoclasts, and also in the IDG-SW3 osteocytic cell line, but not in the MLO-Y4 osteocytic cell line.

In vitro experiments show that activation of the GLP-1R is important in bone metabolism. In one study by Pancheco-Pantoja, GLP-1 increased cell viability and decreased P1NP

secretion in two osteoblastic cell lines, TE-85, and MG-63 (20). In another study, they found that GLP-1 induces c-Fos expression (a gene important in bone cell proliferation and differentiation), in osteoblastic TE-85 cells, with a peak induction after 60 min (45). In mouse osteoblast-like MC3T3-E1 cells, the GLP-1RA, exendin-4, increased proliferation, differentiation, and mineralization through a MAPK pathway (47). Likewise, liraglutide, increased proliferation and differentiation in mouse osteoblast-like MC3T3-E1 cells in one study (47, 48), but in another study, where the cells were cultured in a commercial osteogenic differentiation medium, liraglutide inhibited differentiation, as measured by ALP and osteocalcin in both studies (49). In BMSCs, GLP-1 inhibits adipogenic differentiation, while it promotes osteogenic differentiation (38, 40). Pereira et al. found that both exendin-4 and liraglutide increased osteoclast numbers when added to osteoclast precursor cells derived from mouse bone marrow, while addition to mature osteoclasts reduced the resorbed area (39). In addition, GLP-1RAs have been shown to affect osteoclasts, stimulating their differentiation, but reducing the resorbed area (37), and reducing SOST/sclerostin expression in osteocyte-like MLO-Y4 cells (39).

In vivo, there are multiple studies in rodents establishing GLP-1's role in bone metabolism. In ovariectomized mice, treatment with exendin-4 and liraglutide both had positive effects on trabecular bone, but no effect on cortical bone. Exendin-4, but not liraglutide, caused an increase in calcitonin, and a decrease in serotonin, while both agonists increased osteoclast differentiation, but reduced the resorbed area (37). In a study in streptotozin-induced T1DM mice, short-term treatment with liraglutide had no effect on bone loss, assessed by micro-CT of trabecular microstructure, or bone formation parameters. It did, however, improve tissue material properties (36). Moreover, both GLP-1R KO and double-incretin receptor KO mice show decreased bone quality and strength and reduced cortical area, as well as decreased collagen maturity (93, 103, 104).

Treatment with GLP-1 in normal, insulin-resistant and type-2 diabetic (T2DM) rats, restored the impaired trabecular structure, and while osteocalcin and osteoprotegerin increased in all three groups, RANKL only increased in the T2DM rats (41). In hyperlipidic rats that displayed osteopenia, short-term treatment with GLP-1 or exendin-4 both reversed the decrease in bone mass and quality, while levels of osteocalcin and osteoprotegerin increased (42). Studies in ovariectomized rats have demonstrated that treatment with the GLP-1RA liraglutide improved trabecular number, volume and thickness, and increased aBMD compared to control rats (40), while exendin-4 treatment increased aBMD and BMC measured by DXA, and improved trabecular structure, assessed by micro-CT, and increased bone strength. Gene analysis also revealed that exendin-4 treatment increased the bone formation markers ALP, osteocalcin, and P1NP, while decreasing the bone resorption parameter CTX (43). Liraglutide treatment of the spontaneously diabetic Goto-Kakizaki rats restored the impaired aBMD, and gene analysis showed an increase in bone formation parameters (44), while treatment with exendin-4 in rats with unloading-induced osteoporosis also improved trabecular structure, aBMD and bone strength (38). Exendin-4 treatment of T2DM OLETF rats also increased femoral aBMD, while reducing sclerostin and increasing osteocalcin (39).

Results from human studies are inconsistent. A randomized controlled study by Iepsen et al. showed that liraglutide had beneficial effects on weight-loss-induced bone loss. After an 8-week weight-loss program, 37 women were divided into a control group (19 women) and a group receiving liraglutide (18 women) in the 52-week weight-maintenance phase. BMC, measured by DXA scan, decreased significantly in the control group, but not in the liraglutide group, whereas P1NP increased significantly in the liraglutide group, but not the control group, indicating that the protective effects are mediated by increased bone formation. There was no effect on bone resorption, measured by plasma CTX (35), which is in accordance with an earlier study by Henriksen et al., where subcutaneous GLP-1 treatment had no effect on CTX in seven healthy participants (7).

As GLP-1 analogs are already in use for the treatment of T2DM, there are meta-analyses looking into the correlation between liraglutide and exenatide treatment and the risk of fractures. Studies on T2DM patients treated with GLP-1R agonists found they were not associated with any change in aBMD (105) and the risk of bone fractures was not altered (106). However, in another meta-analysis of randomized controlled trials, a decreased risk of fragility fractures with liraglutide, but an elevated risk with exenatide treatment was found (107).

In conclusion, multiple studies have demonstrated an impact of GLP-1 on bone metabolism involving both an activation of osteoblast function and an inhibition of osteoclasts.

PYY

Peptide YY (PYY) is another hormone secreted from the L-cell in the postprandial state. PYY is often co-secreted with GLP-1 and GLP-2 (108) in proportion to caloric intake, and decreases food intake via appetite-inhibiting actions which involve the hypothalamic arcuate nucleus. It belongs to the pancreatic polypeptide family together with neuropeptide Y (NPY) and pancreatic polypeptide (109), and is secreted as the 36-amino-acid molecular form PYY_{1-36}. After secretion, PYY_{1-36} is degraded by DPP-4 to form PYY_{3-36} (109).

In general, the different PYY molecular forms have different half-lives and act via the four different G protein-coupled Y receptors to which they bind with different affinities. PYY_{1-36} binds to the Y1, Y2, and Y5 receptors, whereas PYY_{3-36} is highly selective for the Y2 receptor. This leads to opposing effects on appetite and possibly also on glucose homeostasis, as reviewed previously (110). PYY_{3-36} is responsible for the anorexigenic actions of PYY, as documented in infusion studies (111, 112) and together with GLP-1, seems to play a role in the decreased food intake and major weight loss seen after the bariatric surgery procedure, Roux-en-Y gastric bypass (111, 113). Meal-induced PYY secretion is blunted in obese participants, but the anorexigenic effect of PYY seems intact (114). COOH-terminally truncated PYY metabolites, $PYY_{1/3-34}$, have recently been described in humans (115). The biological impact of these metabolites remains to be elucidated, but $PYY_{1/3-34}$ has no affinity for the Y2 receptor (115).

TABLE 2 | Current pharmacological treatment of osteoporosis.

Type	Name	Mechanism
ANTI-RESORPTIVE		
Bisphosphonates	Alendronate Ibandronate Risedronate Zolendronic acid	Binds to hydroxyapatite in the extracellular matrix and causes osteoclast cell death by inhibiting the enzyme farnesyl pyrophosphate synthase, disrupting the cytoskeletal structure
Estrogen/SERM	Raloxifene	Binds to the estrogen receptor, which has anti-catabolic effects
Calcitonin		Anti-resorptive effects (in animals)
Antibody mediated	Denosumab	Scavenges RANKL, preventing it from stimulating osteoclast precursor differentiation and maturation
ANABOLIC		
PTH-analog (1-34)	Teriparatide	Hormone replacement therapy: intermittent increase in plasma PTH-levels activates osteoblasts. Daily injections
PTH-analog (1-84)	Natpara	Hormone replacement therapy—long term action

Pharmacological treatment of osteoporosis can be divided into anti-resorptive and anabolic drugs. Bisphosphonates are the most widely used drug for treating osteoporosis. Strontium ranelate is not approved by the FDA, and only restricted use by the EMA, due to severe side-effects, such as increased risk of myocardial infarction and skin reactions. Teriparatide is the only marketed anabolic drug, and mimics endogenous parathyroid hormone (PTH). Intermittent increases in plasma-PTH have anabolic effects, but continuously elevated levels, such as in hyperparathyroidism, have catabolic effects. Several new drugs are being developed, aimed at specific molecules involved in the bone remodeling process, such as inhibitors of cathepsin K (an enzyme secreted from osteoclasts, necessary for the resorption process) and inhibitors of the Wnt/β-catenin-pathway inhibitors sclerostin and dickkopf-1.

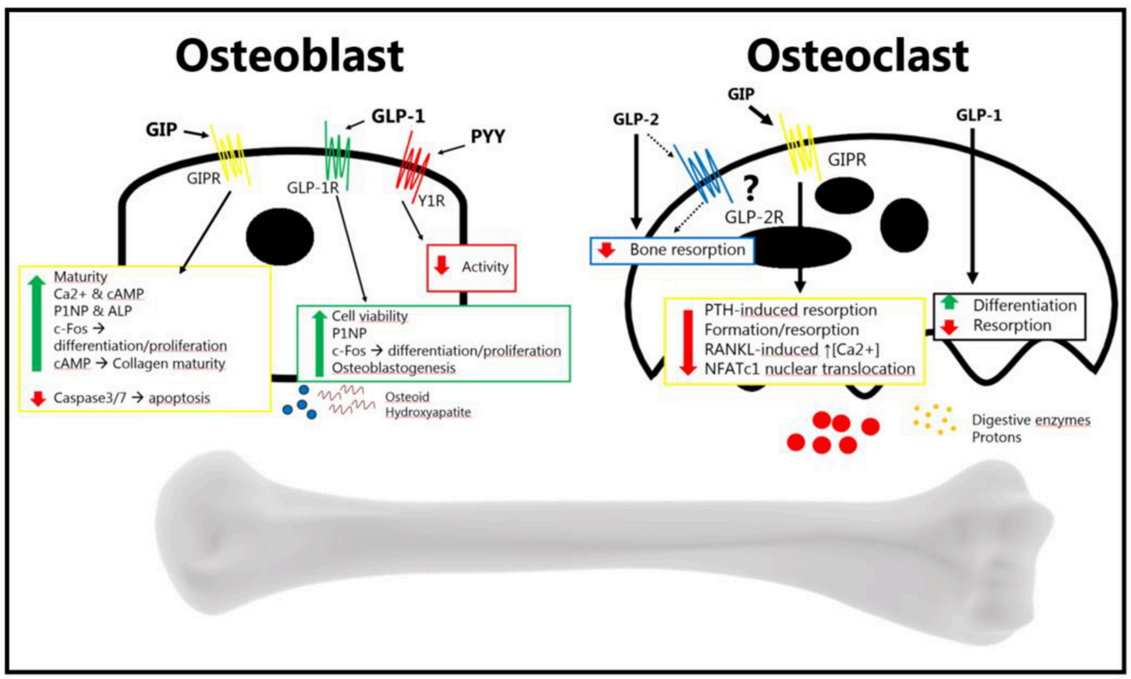

FIGURE 2 | The gut hormones GLP-2, GIP, GLP-1, and PYY, have been shown to affect bone metabolism. The osteoblast increases its activity in response to GIP and GLP-2 (anabolic effects), and decreases its activity in response to PYY (catabolic effects). The exact mechanism of GLP-2 remains to be elucidated. GLP-2 has been shown to be anti-resorptive *in vivo*, an effect which may be direct or indirect. GIP decreases osteoblast activity, and GLP-1 also seems to decrease resorption. PYY's effect, if there is one, has yet to be determined. GLP-2 has been shown to decrease bone resorption, but it is uncertain whether it affects the osteoclast directly. GIP has been shown to affect the osteoclast, reducing bone resorption. This has also been shown for GLP-1, while it has also been shown that it increases differentiation. There is no certain effect of PYY on osteoclasts **(**Bone figure from Somersault18:24, CCBY-NC-SA 4.0).

PYY's Effect on Bones

PYY may exert catabolic effects on bones. Studies in different populations of patients with different kinds of weight alterations have suggested that changes in PYY plasma concentrations modulate bone homeostasis. Elevated fasting PYY was negatively associated with aBMD in pre-menopausal exercising women (116) and in women with anorexia nervosa (50). A negative correlation between elevated PYY and P1NP in young female athletes with amenorrhea (117) further supports an effect of PYY on bone homeostasis. Likewise, the higher postprandial PYY concentration after Roux-en-Y gastric bypass has been suggested to play a role in the marked bone loss that takes place after surgery, and exceeds what is expected from the major weight loss itself. After gastric bypass, CTX increases and this is directly correlated to the changes seen in PYY (51). In contrast, in patients losing weight after gastric banding, another bariatric procedure, both PYY and CTX concentrations were unchanged, supporting a connection between PYY and bone markers (51).

In addition to these associations in human studies, evidence from rodent studies supports a role for PYY in the regulation of bone homeostasis through modulation of both osteoclast and osteoblast activity. The Y1 receptor has been shown on the osteoblast, and PYY might exert suppressive effects on osteoblast activity via these receptors (53). Accordingly, an overproduction of PYY in transgenic mouse models reduced bone mass, whereas PYY knockout mice displayed increased bone mass and strength (54), although the opposite was shown in a study with another PYY knockout model (55). In addition, selective conditional deletion of hypothalamic Y2 receptors in adult mice led to increased bone volume, indicating that the Y2 receptors may also be involved in bone remodeling (56).

Thus, whereas PYY seems to have robust weight-reducing effects, exogenous PYY administration might have a detrimental effect on the bone density.

OSTEOPOROSIS—THERAPEUTIC POSSIBILITIES IN THE GUT-BONE AXIS

Osteoporosis is a bone disease in which the bone becomes more fragile. It represents a growing challenge for health care systems and is also an economic burden, as bone fragility increases the risk of fracture, which is a major cause of morbidity. Fractures often require hospitalization and immobilization, which may cause further complications, and recovery is often slow and incomplete (118, 119). Pathological fractures arise from an imbalance in the bone remodeling process, and are characterized by low bone mass and microarchitectural changes arising from internal factors (primary osteoporosis), such as falling levels of estrogen in postmenopausal women, or external factors (secondary osteoporosis), e.g., malnutrition (120). Pharmacological treatment of primary osteoporosis is divided into two classes of drugs with anti-resorptive and anabolic effects, respectively (121, 122), which is summarized in **Table 2**.

Due to lack of efficiency and intolerable side-effects, current treatment of osteoporosis is limited, and several new drugs are being developed, targeting specific molecules important for bone homeostasis. Examples hereof are inhibitors of sclerostin and dickkopf-1, which are inhibitors of the Wnt signaling pathway, and inhibitors of cathepsin K, which is secreted from osteoclasts and important for the resorption process (121, 122).

The strategy of using GLP-2 for the treatment of osteoporosis has been pursued previously resulting in a series of human studies investigating acute and chronic effects of daily GLP-2 injections on bone remodeling (7, 17–19, 76–78, 123). However, despite a strong inhibition of osteoclast activity and a significant increase in aBMD after 4 months treatment (17), GLP-2 has not reached the market as a new drug for the treatment of osteoporosis, although a GLP-2 analog (teduglutide) was approved by FDA in 2012 for the treatment of SBS because of its beneficial effects on intestinal function (63). As there are already drugs on the market based on GLP-1, the effects on bone metabolism may expand the use of these drugs, or aid the development of drugs more specifically targeted at bone metabolism. GLP-1 based drugs have anorexic effects through their activity in the hypothalamus, which may limit their efficacy as anti-osteoporosis therapy since food intake plays a pivotal role in the maintenance of strong bones. Another potential strategy in the development of drugs based on the hormones in the gut-bone axis, is to target not one, but two or more receptors. This multi-agonism approach may have synergistic effects, and there are studies showing synergistic effects on the treatment of metabolic diseases, such as T2DM (92, 124, 125). Moreover, one study has shown that a GIP-oxyntomodulin hybrid peptide (targeting GIP, GLP-1 and glucagon receptors) had beneficial effects on bone loss in db/db mice with T2DM (36). As all the discussed hormones have anabolic and/or anti-catabolic effects on bone metabolism, all of their cognate receptors are of interest.

In summary, the gut is an important regulator of bone homeostasis, with several gut-derived factors controlling bone formation and resorption (**Figure 2**). The current treatment of osteoporosis is limited, and as GPCRs in general are excellent drug targets, it will be exciting to follow whether novel drugs targeting gut hormone receptors will in the future, reach the market for the treatment of osteoporosis.

AUTHOR CONTRIBUTIONS

SS, KS-J, JW, and MS wrote initial drafts of selected parts of the manuscript. All authors contributed to the writing of the manuscript. MR, BH, and JH assembled, reviewed, and corrected the manuscript.

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Conflict of Interest Statement: MR, BH, and JH are co-founders of Bainan Biotech, focusing on novel treatment of osteoporosis.

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In Pursuit of the Epithelial Mechanosensitivity Mechanisms

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Mechanosensation is critical for normal gastrointestinal (GI) function. Disruption in GI mechanosensation leads to human diseases. Mechanical forces in the GI tract are sensed by specialized mechanosensory cells, as well as non-specialized mechanosensors, like smooth muscle cells. Together, these cellular mechanosensors orchestrate physiologic responses. GI epithelium is at the interface of the body and the environment. It encounters a variety of mechanical forces that range from shear stress due to flow of luminal contents to extrinsic compression due to smooth muscle contraction. Mechanical forces applied to the GI mucosa lead to a large outflow of serotonin, and since serotonin is concentrated in a single type of an epithelial cell, called enterochromaffin cell (ECC), it was assumed that ECC is mechanosensitive. Recent studies show that a subset of ECCs is indeed mechanosensitive and that Piezo2 mechanosensitive ion channels are necessary for coupling force to serotonin release. This review aims to place this mechanism into the larger context of ECC mechanotransduction.

Keywords: enteroendocine cells, mechanosensitivity, mechanosensitive ion channel, serotonin, enteric nervous system

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The gastrointestinal (GI) tract is responsible for sensing luminal chemical and mechanical stimuli (**Figure 1A**) to coordinate the processes of digestion and absorption of ingested nutrients and excretion of wastes. The GI tract also sends signals to the rest of the organism regarding the composition of the luminal contents during digestion, to prepare the metabolic and cardiovascular systems for the flood of absorbed chemicals, and also signals about the lack of nutrients during fasting to assist in adjusting metabolic mechanisms (1). To accomplish these tasks, the lining of the GI tract developed a repertoire of specialized epithelial sensors called enteroendocrine cells (EECs) (2). These cells are distributed sporadically throughout the entire GI tract and serve as beacons of luminal signaling. They sense the nutrients and mechanical stimuli and convert them into physiologically meaningful responses—via secretion of hormones, and local signaling with the intrinsic and extrinsic nerves (3, 4). In turn, EEC disruptions contribute to human diseases that range from gut-centric, such as diarrhea and constipation, to gut-brain axis, such as irritable bowel syndrome (IBS), and system-wide, such as obesity (5).

One of the enteroendocrine cell types, called the enterochromaffin cell (ECC), synthesizes majority of peripheral serotonin (5-hydroxytryptamine, 5-HT). ECC 5-HT has a range of important local effects, like regulation of GI motility and secretion (3, 6), and systemic effects on metabolism during fasting (1). Like other EECs, ECCs are activated by luminal chemical stimuli (7). However, these cells appeared to be different from other EECs when Edith Bülbring found that mechanical pressure applied to the epithelium resulted in release of 5-HT, suggesting that ECCs may also be mechanosensitive (8).

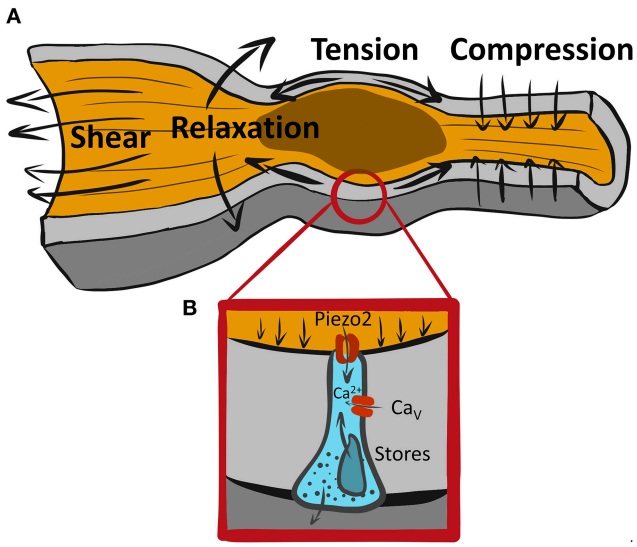

FIGURE 1 | A variety of mechanical forces associated with GI motility may be sensed by mechanosensitive EECs. **(A)** An example of a range of forces associated with propulsive GI motility due to proximal (oral) contraction and distal (aboral) relaxation leading to tension at the wall and shear stress from flowing luminal contents. **(B)** Piezo2+ mechanosensitive EEC senses forces via Piezo2 mechanosensitive ion channels, which produce a "receptor current" leading to intracellular Ca^{2+} increase possibly due amplification by membrane signaling via voltage gated calcium channels (Ca_Vs) and Ca^{2+} release from intracellular stores.

ECC mechanosensitivity was inferred from Dr. Bülbring's experiments that connected luminal pressure to 5-HT release, and has since been demonstrated in other studies in animals (9) and humans (10). However, since the enteric nervous system has complex multicellular organization, and multiple cell types are mechanosensitive, it was important to determine whether ECCs are intrinsically mechanosensitive, or if they respond to signals from other mechanosensitive cells in the GI tract.

MECHANOSENSITIVITY OF IMMORTALIZED 5-HT SECRETING NEUROENDOCRINE CELLS

ECCs make up ∼1% of the epithelium, and their random distribution makes them difficult to purify and identify. Further, epithelial cells are constantly turned over in a balanced process of stem cell replication and anoikis, or attachment dependent apoptosis. Therefore, isolated epithelial cells have very short lifespans, so primary cultures of ECCs have a limited shelf life. Because of these issues, first studies that examined ECC mechanosensitivity used immortalized 5-HT secreting cells from neuroendocrine tumors (11). A pancreatic neuroendocrine cell, called BON, was cultured and mechanically stimulated by rotational shaking, which resulted in 5-HT release (12). This 5-HT release depended on intracellular Ca^{2+} increase that was driven by activation of $G_{\alpha q}$ leading to release of Ca^{2+} from intracellular stores. While $G_{\alpha q}$ is not known to be mechanosensitive, $G_{\alpha q}$ activation in BON cells required association with caveolins, which are mechanosensitive (13). Mechanical stimulation of BON cells by touching or rotational shaking also led to ATP release and autocrine activation of P2X and P2Y receptors (14). A follow up study showed involvement of adenosine receptors in BON cell mechanotransduction (15), and mechanotransduction of a different cancer cell line, KRJ-I (16).

Specialized epithelial mechanosensors are distributed throughout our bodies, and include Merkel cells in the skin required for touch (17) and hair cells in the ear required for hearing (18). Recent work showed that Merkel cells rely on a mechanosensitive ion channel called Piezo2 (*Piezo* is Greek for "squeeze" or "press"). There are developmental and functional similarities between Merkel and ECCs—they have multiple common developmental transcription factors, are both mechanosensitive and secrete 5-HT (19). So, we examined whether Piezo2 channels may contribute to ECC mechanosensitivity. We used another cancer-derived 5-HT secreting neuroendocrine cell, called QGP-1, and found that Piezo2 was expressed (20). When these cells were mechanically stimulated by direct membrane displacement, they produced a force-induced ionic current that had biophysical properties of Piezo2—rapid onset and inactivation, and steep mechanosensitivity without rectification. Further, when we grew QGP-1 cells on flexible membranes and stretched them, we found that they released 5-HT. This mechanosensitive 5-HT release was inhibited by a variety of mechanosensitive and Piezo ion channel blockers and importantly by Piezo2 siRNA but not non-targeted (NT) siRNA (20). In all, studies using immortalized neuroendocrine cell lines have and continue to provide valuable information on EEC mechanosensitivity (11). The data in 5-HT releasing neuroendocrine cell lines showed that they are mechanosensitive and that they employ a range of mechanisms of mechanotransduction to convert force into 5-HT release—including G-protein coupled pathways as well as ionic pathways. The results on Piezo2 ion channel were particularly intriguing to us, because this ion channel is established to be mechanosensitive (21), unlike the other described molecules, and it was shown to be a primary mechanosensor critical for mechanosensitivity in other epithelial mechanosensors.

MECHANOSENSITIVITY OF PRIMARY ECCs

What Is the Primary Mechanosensor?

A critical question is whether primary ECCs are mechanosensitive. Chin et al. purified ECCs from patients with inflammatory bowel disease and grew them on flexible substrates and when they stretched them, they found 5-HT release that depended on adenosine signaling (16). However, the nature of the mechanosensor upstream of this mechanism remained unclear. We pursued the hypothesis that Piezo2 channels were important for ECC mechanosensitivity (20, 22, 23). We examined Piezo2 expression in human jejunum and transgenic reporter and lineage traced mice and found that Piezo2 was expressed in ECCs but that not all ECCs expressed Piezo2 (20, 22). Further,

while majority of Piezo2 was present in 5-HT positive cells, some Piezo2+ cells were 5-HT negative (22). So, we explored whether EECs were mechanically sensitive (22). Using electrophysiology, we found that more than 50% of EECs had mechanosensitive ionic currents, unlike other, likely epithelial cells, in primary culture. Membrane displacement produced fast ionic currents—they activated with rapid membrane displacement within milliseconds and inactivated almost as rapidly—within few dozen milliseconds. EEC mechanosensitive currents were steeply mechanosensitive, going from off to on within 2 μm membrane displacement, and they were non-rectifying. These were biophysical properties of Piezo2 channels, so we used pharmacology and knockdown to determine whether the EEC mechanosensitive currents were indeed carried by Piezo2. However, given the speed of the mechanosensitive currents, we wondered how those fast currents could lead to 5-HT release that lasts for seconds? Using Ca^{2+} imaging, we found that in isolated mechanosensitive EECs both shear stress and membrane displacement led to a fast rise in intracellular Ca^{2+} but return to baseline Ca^{2+} took tens of seconds, regardless of whether ECCs were stimulated very briefly (50 ms) or more slowly (20 s). Mechanosensitive increase in Ca^{2+} was dependent on Piezo2 and was required for 5-HT release, which we measured in single cells using biosensors. As with intracellular Ca^{2+} increase, mechanically stimulated 5-HT release lasted for several seconds after even one rapid (50 ms) stimulation.

THERE ARE MANY REMAINING QUESTIONS

What Are the Mechanotransduction Pathways That Link Piezo2 and 5-HT Release?

Considering how fast Piezo2 inactivates, the duration of Ca^{2+} increase with mechanical force is interesting, but not surprising. Amplification of the rapid "receptor current," such as Piezo2, is common in sensory neurobiology, and it occurs in other epithelial mechanosensors, such as Merkel cells and hair cells in the ear (24) (**Figure 1B**). Cells frequently use Ca^{2+} signaling to regulate the amplitude and duration of the response to receptor current. Though it is currently unclear, Piezo2 receptor current, which is non-selective for Na^{+} and Ca^{2+}, may have three possible roles: (1) it could bring in some Ca^{2+} which would initiate 5-HT release that may stimulate further secretion by autocrine mechanism, (2) it could depolarize the ECC membrane and lead to activation of sodium or calcium voltage-gated channels (7, 25, 26), and/or (3) it could bring in Ca^{2+} which may activate Ca^{2+} activated Ca^{2+} release. Ca^{2+} handling mechanisms are important in ECC function, both in the context of chemo- and mechano-sensation. Thus, several types of voltage-gated Ca^{2+} channels are found by expression and functional analysis using pharmacologic blockers and voltage-clamp in both immortalized neuroendocrine cells and primary EECs. These include L-type ($Ca_V1.3$, Cacna1d), T-type ($Ca_V3.2$, Cacna1h), and P/Q-type ($Ca_V2.1$, Cacna1a) (7, 26–28). Both L-type (26) and P/Q-type (7) channels, but interestingly not the highly expressed T-type Ca_V channels, have found their functional relevance in chemotransduction, but not all studies agree on the roles of these channels in chemotransduction. Future work will need to determine whether Ca_V channels are involved in ECC mechanotransduction.

Are Human EECs Mechanosensitive?

As I describe above, multiple studies examined mechanosensitivity of human immortalized neuroendocrine cells. But we still have limited knowledge about human enteroendocrine cell mechanosensitivity. We know that mechanical stimulation of ECCs leads to 5-HT release (16), but we do not know whether increased 5-HT is due to increased secretion or decreased reuptake (10). Purified human ECCs from patients with inflammatory bowel disease were grown in primary culture on flexible substrates lead to stretch-dependent 5-HT release that depend on activation of ADORAs (16). However, since adenosine and ATP are frequently released by mechanical stimuli by non-specialized mechanosensitive cells (29), we do not know whether 5-HT release from these cells is due to their being specialized mechanosensors. So, while Piezo2 channels were found in human jejunum ECCs (20), but we do not know whether these cells are mechanosensitive. Progress in the field of human ECC physiology is hampered by the same barriers that limited the studies of ECCs from animal models—they are sparse and primary cultures do not survive long term. Yet, significant progress is being made in human epithelial cell models and culturing techniques, suggesting that intellectual progress on human EEC physiology is not far behind.

Are ECCs the Only Mechanosensitive EECs?

A recent study in drosophila showed that a population of EEC precursors express Piezo channels (drosophila has only one Piezo gene) and regulate the density of mechanosensitive EECs which are important to respond to luminal filling or compression due to muscle contractions (30). Our recent work shows that Piezo2 is mostly in ECCs, but both by immunohistochemistry and 5-HT release measurements, not all mechanosensitive EECs are ECCs (22). This is not surprising, as recent studies show that delineation between different EEC subtypes that we are used to is less accurate than seeing ECCs as a part of a continuous EEC spectrum, suggesting that the differences between EEC subtypes may be subtle in mice (31) and humans (32). Further, EEC phenotype is not stable, since expression of both signaling molecules and receptors varies along with cell migration through the crypt-villus axis (33). These circumstantial clues suggest that mechanosensitive ECCs may release bioactive substances along with 5-HT, and in addition to ECCs, other EECs may be mechanosensitive, and finally that mechanotransduction elements may be differentially expressed during EEC development. For us to understand EEC roles in physiology, we first must understand the repertoire of mechanosensitive EECs and their products.

What Are the Physiologically Relevant Forces in the Epithelium?

Studies aiming to understand GI mechanotransduction at the single cell level use a variety of mechanostimulation techniques. Some notable examples include shear stress (34), direct membrane displacement by probes (20, 22) rotational shaking (12), pressure clamp (34), and stretch of flexible substrates (16, 20). Each of these techniques has its advantages and disadvantages, so it is important to use multiple techniques on the same preparation. Single EEC mechanosensitivity was tested using rotational shaking (12), membrane displacement (22), and stretch via flexible membranes (16, 20), and these stimuli produced responses, as measured by 5-HT, intracellular Ca^{2+} and membrane currents as read outs. However, it is not always clear what forces the mechanosensitive cells encounter (**Figure 1A**). Specialized mechanosensitive cells are built to respond to acute mechanical stimuli, but they also reside in an environment that has resting mechanical forces. The gut wall is a composite material with different layers (mucosa, submucosa, muscularis, and serosa) having different mechanical properties. At the tissue scale, mucosa has non-trivial resting mechanical energy, which is placed within the confines of a mechanically stiff muscularis layer (35). The situation is no less complex within the epithelium. For example, during the peristaltic reflex (**Figure 1A**) an epithelial cell, such as EEC, likely encounters several different forces. It feels the compressive force from proximal muscle contraction against luminal contents, stretch due to distal relaxation and shear stress from the flow of luminal contents. This means that an ECC at the tip of the villus likely experiences shear and compression, while an ECC in the crypt experiences compression and stretch, but much less shear stress. At the cellular scale, EECs reside within an epithelial monolayer, which is a crowded setting, and the resting forces that exist due to crowding (36), and on a larger scale, the villi have a resting stiffness, which provides background mechanical force for the epithelium (35). So, acute forces from GI physiologic processes need to be detected from above the resting mechanical background. To make progress, we need to understand not just the mechanisms of EEC mechanotransduction, but also the mechanical environment within which physiologic forces exist. Further, since there are several mechanosensitive cell types in the GI tract which are arranged in complex mechanosensory circuits, the nature and location of force to each of the mechanosensors is integrated to obtain physiologic effect. For example, do ECCs respond to luminal forces, such as secretion-driven volume expansion and shear stress, or to muscularis driven contraction compressing the mucosa, or both?

In conclusion, recent advances in ECC physiology and mechanosensitivity have uncovered specific mechanotransduction pathways that couple GI forces to 5-HT release. This progress will lead to better understanding of ECC contributions to GI physiology, and whether ECC mechanosensation contributes to GI pathophysiology. However, many important questions remain, including understanding of the specifics of mechanism of mechanotransduction in animal models and humans, the repertoire of mechanosensitive EECCs, and how ECC mechanosensitivity fits into the context of GI mechanobiology.

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AB planned, wrote, and edited the paper.

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REVIEW
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The Regulation of Peripheral Metabolism by Gut-Derived Hormones

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Enteroendocrine cells lining the gut epithelium constitute the largest endocrine organ in the body and secrete over 20 different hormones in response to cues from ingested foods and changes in nutritional status. Not only do these hormones convey signals from the gut to the brain via the gut-brain axis, they also act directly on metabolically important peripheral targets in a highly concerted fashion to maintain energy balance and glucose homeostasis. Gut-derived hormones released during fasting tend to be orexigenic and have hyperglycaemic potential. Conversely, gut hormones secreted postprandially generally promote satiety and facilitate glucose clearance. Although some of the metabolic benefits conferred by bariatric surgeries have been ascribed to changes in the secretory profiles of various gut hormones, the therapeutic potential of the enteroendocrine system as a viable target against metabolic diseases remain largely underexploited, except for incretin-mimetics. This review provides a brief overview of the physiological importance and highlights the therapeutic potential of the following gut hormones: serotonin, glucose-dependent insulinotropic peptide, glucagon-like peptide 1, oxyntomodulin, peptide YY, insulin-like peptide 5, and ghrelin.

Keywords: GLP-1, PYY, serotonin, GIP-glucose-dependent insulinotropic peptide, oxyntomodulin, ghrelin, enteroendocine cells, insulin-like peptide 5 (INSL5)

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INTRODUCTION

Gut enteroendocrine cells (EECs) are specialized secretory cells that are sparsely scattered throughout the mucosal epithelium of the gastrointestinal (GI) tract and which constitute the largest endocrine organ by mass in the body (1). EECs have the capacity to "sample" luminal contents on the apical membrane, and collectively release more than 20 different hormones basolaterally in response to a variety of stimuli. With each having their own specialized functions, EECs have been historically characterized by their hormonal profiles, such as glucagon-like peptide 1 (GLP-1)- and peptide YY (PYY)-secreting L-cells and serotonin (5-hydroxytryptamine, 5-HT)-secreting enterochromaffin (EC) cells. It is now accepted that there are vast overlaps in the secretory profiles of EECs (2) and the "one cell type, one hormone" dogma is widely rejected. Studies using transgenic mice expressing fluorescent reporter proteins driven by promoters of different gut hormones revealed that multiple hormones can be simultaneously expressed by an individual EEC (3, 4) while high-resolution microscopy shows that these different hormones are packaged into separate vesicles within the EEC (5–7). Expression of EEC hormones are also regionally distinct, as

many gut hormones are confined to specific regions of the gut, while a subset, such as 5-HT and somatostatin, are present throughout the GI tract (8, 9). Enteroendocrine hormones are implicated in a wide range of physiological functions including gastrointestinal motility, appetite control, and glucose homeostasis (10). Mounting evidence demonstrates the importance of gut hormones in regulating peripheral metabolism in health and disease and as a result, a myriad of therapeutics against metabolic diseases that are based on the actions of specific gut hormones are currently under clinical development (11–13). As such, it is timely to review the literature regarding the metabolic actions of these gut hormones: serotonin, glucose-dependent insulinotropic peptide, glucagon-like peptide-1 (GLP-1), oxyntomodulin, peptide YY (PYY) and ghrelin. We also discuss the metabolic actions of insulin-like peptide 5, a recently characterized gut hormone that are co-secreted with GLP-1 and PYY.

SEROTONIN

Serotonin (5-HT) is produced by enterochromaffin (EC) cells, which constitute ∼50% of the total EEC population and are scattered throughout the length of the gut, from the stomach to the distal colon (2, 8). Although better known for its actions in the CNS, more than 90% of total body 5-HT is synthesized by EC cells, the majority of this being stored in platelets (14, 15). Tryptophan hydroxylase 1 (TPH1) is the rate-limiting enzyme of 5-HT synthesis in specific non-neuronal cells and its expression in the gut mucosa is limited to EC cells. EC cells have the capacity to sense a wide range of stimuli present in the gut lumen such as glucose and fructose (16, 17), the medium chain fatty acid, lauric acid (18), various tastants and olfactants (19), and to secrete 5-HT in response. 5-HT secretion from EC cells is also regulated by mechanical stimuli (20), and neural and endocrine input such as adrenergic stimulation and GABA and somatostatin inhibition (21). In addition, microbial metabolite signals from the gut microbiome also augment colonic EC cell density, 5-HT secretion and circulating 5-HT levels (22).

Although traditionally regarded as a regulator for gastric motility (23–25) and more recently, a mediator in the pathogenesis of inflammatory intestinal disorders (14, 26), mounting evidence highlights gut-derived 5-HT as a modulator of peripheral metabolism (27, 28). Under fasting conditions, gut-derived 5-HT, together with glucagon, markedly increases hepatic glucose output, a main driver of fasting euglycaemia, by increasing hepatic gluconeogenesis and glycogenolysis (29), while inhibiting glucose uptake and glycogen synthesis in the liver (30). In conjunction, 5-HT promotes lipolysis within white adipocytes to liberate free fatty acids (FFAs) and glycerol (30) as key substrates for hepatic gluconeogenesis, and further enhance hepatic glucose output. Moreover, gut-derived 5-HT promotes energy conservation and weight gain by reducing energy expenditure, via actions to attenuate thermogenesis in brown adipose tissue (31) and inhibit the browning of white adipose tissue (32).

Gut-derived 5-HT also attenuates the release of several metabolically important blood glucose-lowering chemokines, such as adiponectin from adipose tissue (33), and bone-derived osteocalcin and lipocalin 2 (34–36), through inhibition of osteoblast proliferation (37). Significantly elevated mucosal *TPH1* expression in obese humans (38, 39) and elevated levels of circulating 5-HT in individuals with type 2 diabetes (T2D) (40–42) or obesity (38) has been reported. Inhibition of intestinal TPH1 in mice, through tissue-specific ablation or pharmacological inhibition, conveys protection from high-fat diet (HFD)-induced dyslipidaemia and glucose intolerance (30–32). This confirms a causative role of elevated gut-derived 5-HT as a driver of metabolic dysfunction. TPH1 inhibition also protects mice from diet-induced obesity (DIO) (31). However, despite clear evidence that EC cell-derived 5-HT negatively impacts energy balance and glucose homeostasis, the underlying causes of elevated 5-HT levels with obesity and T2D remain unclear. Likely drivers of increased circulating 5-HT are increased density or glucose-sensitivity of duodenal EC cells, as evidenced in obese human duodenal EC cells (38), however molecular mechanisms underlying this are not understood. Due to the heterogeneity in 5-HT receptors across many tissues (43), targeting 5-HT receptor signaling pathways may not be a viable therapeutic target for treatment of metabolic disease.

GLUCOSE-DEPENDENT INSULINOTROPIC PEPTIDE

Glucose-dependent Insulinotropic Peptide (GIP) is a 42-amino acid peptide hormone produced by K cells located primarily in the proximal small intestine (44). GIP is secreted in response to nutrient stimulation and exerts its actions by binding to the GIP receptor (GIPR) expressed by pancreatic islet cells (45), adipocytes (46), bone cells (47), and the CNS (48). Circulating GIP is rapidly degraded by dipeptidyl peptidase IV (DPP4), a serine protease that is widely expressed throughout the body, especially in endothelial cells (49). The insulinotropic effect of GIP, together with GLP-1, accounts for more than 70% of postprandial insulin secretion (50). GIP also increases insulin biosynthesis (49), promotes β-cell proliferation and inhibits β-cell apoptosis (51). The insulinotropic effects of GIP are dramatically attenuated in T2D patients (52, 53), and this is believed to be a major contributing factor to impaired postprandial insulin secretion in these individuals. Moreover, the insulinotropic potency of GIP is markedly reduced in non-diabetic, first-degree relatives of T2D patients (54), suggesting altered GIP signaling could be one of the many predisposing factors for T2D later in life. While the mechanism underlying the diminished insulin response to GIP in T2D has not yet been fully elucidated, receptor downregulation (55) and desensitization (56) have been suggested as potential causes. Although GIP only stimulates glucagon secretion under hypo- and euglycaemic conditions in healthy individuals (57), its glucagonotropic effect is exaggerated in T2D patients during hyperglycaemia (58). This further worsens glycaemic control in these patients, and in combination with the reduced insulinotropic potency renders GIP an undesirable therapeutic target for T2D treatment.

The anabolic properties of GIP closely resemble those of insulin, as it promotes lipid uptake and inhibits lipolysis in adipocytes (59). Several studies have reported elevated GIP levels in obese humans (60, 61). Elevated GIP levels and duodenal K cell hyperplasia (62) have also been reported in HFD-treated mice, while *Gipr* deficiency protects mice from HFD-, leptin deficiency- or ovariectomy-induced weight gain (63, 64). GIP also induces osteopontin expression in adipocytes (65), an adipokine associated with obesity-related systemic low grade inflammation (66, 67). Adipocyte-specific *Gipr* ablation protects mice from HFD-induced insulin resistance and hepatic steatosis, potentially by reducing circulating levels of pro-inflammatory cytokines (68). However, the obesogenic effects of GIP are only apparent during nutrient excess, as chow-fed *Gipr* and *Gip* knockout animals are of similar weight as their wild type counterparts (69). The role of GIP in energy balance is further complicated by paradoxical findings that mice overexpressing *Gip* were leaner than wild type controls, when fed either a standard-chow or HFD (70). Such observation could be attributed to the anti-apoptotic effect of GIP on osteoblasts (71), as osteoblast-derived hormones such as osteocalcin and lipocalcin 2 are implicated in regulating peripheral metabolism and modulate food intake (36, 72). Furthermore, powerful evidence has emerged to show that GIPR signaling can enhance GLP-1-induced weight loss (11, 73).

GLUCAGON-LIKE PEPTIDE 1

Glucagon-like Peptide 1 (GLP-1) is an incretin hormone secreted by enteroendocrine L cells upon ingestion of nutrients, including glucose (74), and typically within 10–15 min into the postprandial period (75). GLP-1 is subjected to rapid degradation by DPP4 (76) and acts via the GLP-1 receptor (GLP-1R) expressed on a myriad of target tissues (75). GLP-1 plays a key role in maintaining glucose homeostasis, as it markedly increases glucose-stimulated insulin secretion (GSIS) (77) and attenuates hepatic glucose production, independent of its effect on pancreatic islets (78, 79). There is growing appreciation that a considerable portion of the glucose-lowering effect of GLP-1 is underscored by its inhibitory effect on gastric motility (80–83) and its glucagonostatic action (84, 85), which are preserved in obese and T2D patients (86, 87). Unlike GIP, the potent insulinotropic effect of GLP-1 is predominantly preserved in T2D patients and, thus, has led to the development of GLP-1-based therapies for preserving blood glucose control in individuals with T2D.

In addition to its multifaceted glucose-lowering effect, GLP-1 regulates energy balance and adiposity through its effects on satiety and appetite. The acute anorectic effect of GLP-1 is mediated by GLP-1R located on vagal afferents (88), which relays the signal to appetite control centers, namely the NTS in the brainstem, to reduce food intake (89) (**Figure 1**). GLP-1R are also widely expressed in brainstem and hypothalamic regions implicated in appetite control (90). In humans, acute administration of pharmacological doses of GLP-1 significantly induce satiety and reduce food intake (91–93). Furthermore, exaggerated postprandial GLP-1 response is believed to contribute to the increased satiety reported by many gastric-bypass surgery patients (94–96). However, a recent clinical study reported that the infusion of exendin 9-39, a GLP-1R antagonist, did not affect *ad libitum* food intake in post-RYGB patients, although the authors also reported a concomitant increase in plasma levels of the anorexigenic hormone PYY (discussed below), which might offset the orexigenic effect of GLP-1R antagonism (94). The DPP4-resistant GLP-1R agonist, liraglutide, is now in clinical use as a weight-loss therapeutic in obese/overweight individuals (97). GLP-1 is also implicated in regulating hedonic eating through GLP-1Rs located elsewhere in the brainstem (98–100). Peripherally administered GLP-1R agonists may also act directly on GLP-1R at other sites in the brain, notably circumventricular organs and some hypothalamic regions with fenestrated capillaries (101–103). Indeed, Liraglutide can directly activate anorectic POMC/CART neurons in rodents and thus, indirectly inhibit orexigenic AgRP/NPY neurons in the arcuate nucleus (ARC) to reduce food intake (101). As endogenous GLP-1 has a very short half-life, these central actions are likely to be more relevant during therapeutic use of DPP4-resistant GLP-1R analogs, or in post-gastric bypass surgeries, in which GLP-1 "equivalent" levels, or postprandial GLP-1, respectively, are augmented and sufficient to elicit anorectic responses at these CNS targets.

OXYNTOMODULIN

Oxyntomodulin (OXM) is a 37-amino acid peptide that contains the entire amino acid sequence of glucagon (104) and is co-secreted with GLP-1 by enteroendocrine L cells at an equimolar ratio (105). Although an endogenous OXM receptor has not been identified, OXM exerts weak agonist activity on GLP-1R (106) and the glucagon receptor (GCGR) (107). Nevertheless, pharmacological levels of OXM (sufficient to activate GLP-1R and GCGR) have shown anti-obesity effects in humans, by significantly reducing appetite (108, 109) and increasing energy expenditure (110). In addition, OXM treatment improved glucose tolerance in high-fat fed mice by potentiating GSIS (111), in a glucose-dependent manner (112), and has an anti-apoptotic effect on β cells (112). OXM infusion significantly reduced glycaemic excursion by augmenting GSIS in obese subjects with or without T2D (113). Such observations prompted the investigation into the potential metabolic benefits of GLP-1R and GCGR co-activation (114, 115), which led to the subsequent development of GLP-1R/GCGR co-agonists (73, 116) and, later, GIPR/GLP-1R/GCGR tri-agonists (117). These agonists have shown impressive anti-obesity effects in preclinical models and are currently being evaluated in phase 2 clinical trials (118).

PEPTIDE YY

Peptide YY (PYY) is co-localized with GLP-1 in enteroendocrine L cells (7, 119) and is co-released with GLP-1 postprandially,

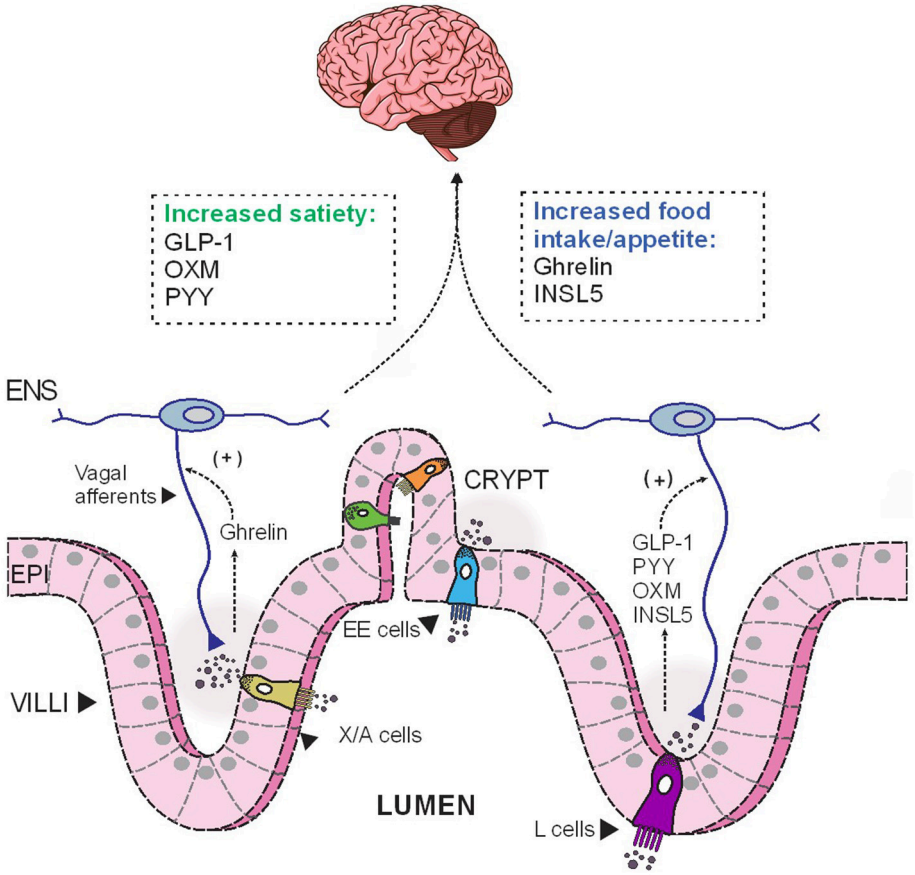

FIGURE 1 | The opposing effects of anorectic and orexigenic gut hormones on food intake. Depending on the luminal stimulation, EE cells release different hormones basolaterally, which then diffuse across the lamina propria to act on their corresponding receptors expressed on nerves endings of vagal and enteric afferents. These hormonal cues are subsequently relayed to the CNS to modulate food intake. EE, enteroendocrine; ENS, enteric nervous system; EPI, epithelial cells; INSL5, insulin-like peptide 5; GLP-1, glucagon-like peptide 1; OXM, oxyntomodulin; PYY, peptide YY.

in proportion to caloric intake (119, 120). In contrast to GLP-1, which is present in sufficient amount in the duodenum to account for the immediate postprandial surge, PYY abundance is very low in the upper gut and increases distally from the ileum toward to colon (121, 122). Thus, postprandial PYY release under normal physiological conditions is likely to be mediated through paracrine and neural mechanisms (123). An exaggerated postprandial PYY response is observed in gastric bypass patients, and is likely attributed to the increased flow of nutrients into the PYY-rich distal gut, which can directly stimulate L cells (124, 125). Human PYY circulates in two active forms: PYY_{1-36} and PYY_{3-36}, the latter being an active cleavage product of the former by DPP4 (126). Both are key mediators of the "ileal brake," a local feedback mechanism triggered by the arrival of nutrients in the ileum that inhibits gastric and pancreatic secretions and proximal intestinal motility (127). The physiological effects of PYY are mediated through a family of NPY receptors (termed Y1, Y2, Y3, Y4, and Y5), which are differentially expressed in a wide range of tissue including enterocytes, myenteric and submucosal neurons and extrinsic primary afferent nerve fibers (123).

Exogenous PYY administration significantly reduces food intake in both obese and lean subjects (128, 129). *Pyy*-deficient mice are hyperphagic and obese (130) while *Pyy* overexpression protects mice against obesity induced by HFDs or leptin deficiency (131). Although the "ileal brake" mechanism contributes to its satiating effect (132), PYY_{3-36} induces satiety primarily by targeting the hypothalamus. The role of PYY as a satiety hormone has been debated, as several independent research groups did not reproduce the anorectic effect in humans reported in the original study by Batterham et al. (133). Moreover, due to its nauseating effect at higher doses (134–136), PYY has not been pursued as an anti-obesity target.

PYY infusion in humans had limited effects on plasma glucose, insulin or glucagon levels on its own (128, 137), nor did it affect glucose excursion and insulin levels during intravenous (138) or oral glucose challenge (136). PYY has trophic effects on pancreatic β cells (139), but such effects are believed to be mediated by islet-derived, rather than gut-derived PYY (140). However, as postprandial PYY levels after gastric bypass surgeries are elevated several folds, it may be possible for gut-derived PYY to exert protective effect on β cells in these settings.

GHRELIN

Ghrelin is an orexigenic hormone secreted by X/A cells present in the mucosa throughout the length of the GI tract, with the highest abundance in the gastric fundus. Circulating ghrelin is significantly elevated during fasting and attenuated upon meal initiation. Post-translational acylation of the ghrelin peptide by ghrelin O-acyl-transferase (GOAT) is crucial for its activity at its endogenous receptor, growth hormone (GH) secretagogue receptor (GHSR1a) (13). GHSR1a is highly expressed in the CNS and is capable of stimulating GH release from the anterior pituitary (13), and lower levels of expression are found in the periphery including the small intestine and pancreatic islets (141). Exogenous ghrelin reliably increases food intake in various species, including humans (142). The orexigenic action of ghrelin is mediated through direct stimulation of the orexigenic AgRP/NPY neurons and concomitant inhibition of the anorectic POMC/CART neurons in the ARC (143, 144). Weight loss achieved through caloric restriction is accompanied by marked elevation in circulating ghrelin (145), which increases feeding drive and has therefore been ascribed as a natural defense against weight loss. Ghrelin is also an anabolic hormone that drives lipogenesis, independent of its effect on appetite (146). Altogether, the orexigenic and anabolic properties of ghrelin renders the ghrelin-GOAT-GHSR1a axis an attractive anti-obesity target. Pharmacological blockade of GOAT or GHSR1a have yielded promising results in preclinical models of obesity (147–150). However, genetic disruption of different components of the ghrelin-GOAT-GHSR1a axis in mice did not have the anticipated anorectic or anti-obesity effects (151–154). Neither *Ghrelin* nor *GOAT* deficiency rescue the obese and hyperphagic phenotype of *ob/ob* mice (152, 155). As such, these data indicate a dispensable role for ghrelin in the regulation of feeding and bodyweight, and that the role of ghrelin in increasing feeding drive may be limited to fasting conditions.

Contrary to its limited role in feeding behavior, ghrelin is a key regulator of glucose homeostasis. Exogenous ghrelin markedly increases blood glucose levels in humans, while genetic ablation of ghrelin or its receptor improve glucose tolerance in HFD-fed and *ob/ob* mice (152, 156). Ghrelin receptor signaling, specifically in hypothalamic AgRP/NPY neurons, is a critical countermeasure to prevent hypoglycaemia (143). Mice with attenuated ghrelin signaling, due to *GOAT*-deficiency or ghrelin cell ablation, have a blunted counter-regulatory GH response, and display profound fasting-induced hypoglycaemia (157, 158). Ghrelin protects against hypoglycaemia by triggering the direct release of GH from the anterior pituitary (159), increasing glucagon secretion (160) and inhibiting insulin secretion (161, 162). Ghrelin can protect mice from hypoglycaemia in the absence of intact GCGR signaling (163). Thus, ghrelin may be a potential treatment for acute insulin-induced hypoglycaemia in type 1 diabetes patients.

INSULIN-LIKE PEPTIDE 5

Insulin-like peptide 5 (INSL-5) is predominantly expressed in the brain and colonic L cells (164, 165), with immunohistochemical staining and FACS analysis revealing that INSL-5 is overwhelmingly co-expressed with GLP-1 (164). Belonging

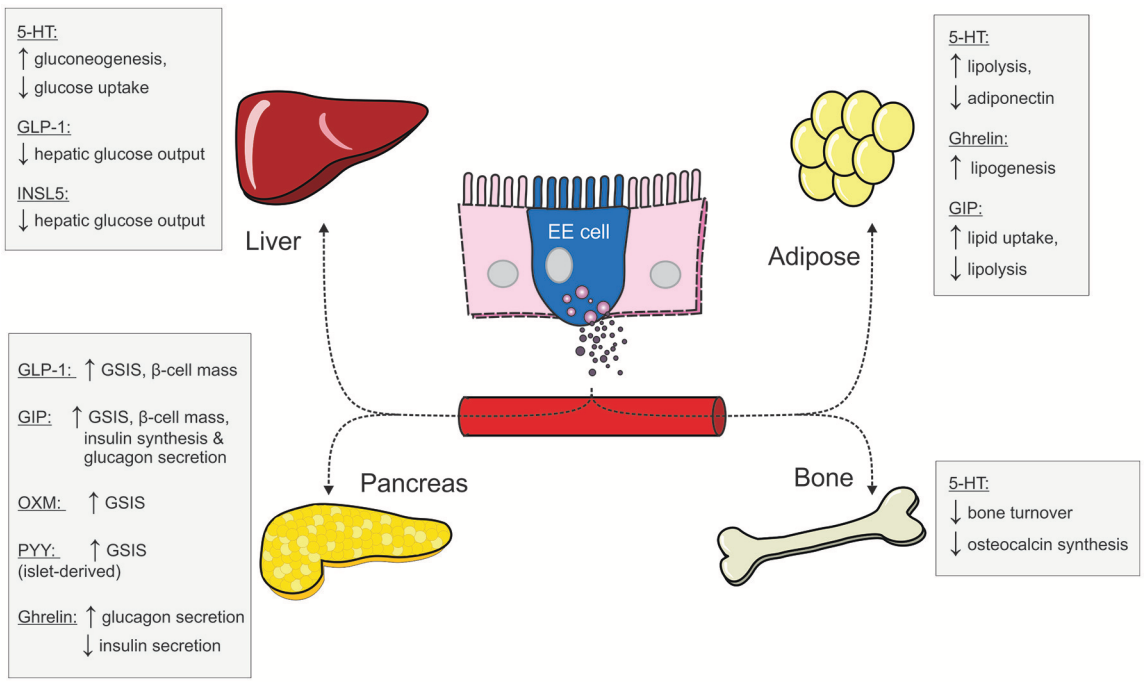

FIGURE 2 | The peripheral metabolic effects of different gut hormones (5-HT, serotonin; EE, enteroendocrine; GIP, glucose-dependent insulinotropic hormone; GLP-1, glucagon-like peptide 1; INSL5, insulin-like peptide 5; OXM, oxyntomodulin; PYY, peptide YY; GSIS, glucose-stimulated insulin secretion).

to the Relaxin-peptide superfamily, INSL-5 has recently been identified as anorexigenic hormone. Secreted INSL-5 acts on the Relaxin/Insulin-like family peptide receptor 4 (RXFP4) (166), which is expressed along the GI tract, the nodose ganglion and the enteric nervous system (164), and inhibits adenylyl cyclase activity (167). Intraperitoneal, but not intracerebroventricular, administration of INSL-5 dose-dependently increases food intake in mice, indicating the peptide may exert its orexigenic effect by acting on peripheral targets, rather than via the CNS (164).

Strong evidence supports the role of INSL-5 as an energy sensor within the colon. Colonic *Insl5* and plasma INSL-5 levels are elevated during fasting in calorie-restricted mice and normalize upon refeeding (164). Increased colonic expression of *Isnl5* is also observed in germ-free (GF) mice, which lack a gut microbiome (168) and microbial-produced colonic short-chain fatty acids (SCFAs). As a consequence, GF mice have energy-depleted colonocytes due to the absence of their SCFA energy source, butyrate (169). Indeed, the introduction of a functional gut microbiome, which increases luminal SCFA availability, leads to reduced *Insl5* expression, in a manner similar to refeeding calorie-restricted mice (169). The role of INSL-5 as an energy sensor within the colon is not restricted to the availability of SCFAs, as *Insl5* expression in GF mice can also be reduced following HFD consumption, in which unabsorbed lipids provide an alternative energy source to colonocytes (168). As such, INSL-5 may serve as an important link between the gut microbiota and host in the context of metabolism.

The effect of INSL-5 on glucose homeostasis is less clear. While it was initially reported that mice deficient in *Insl5* were mildly glucose-intolerant (170), this appears to be age (170) and strain-dependent (164, 168). $Insl5^{-/-}$ mice have impaired intraperitoneal glucose tolerance but superior insulin sensitivity and moderately reduced hepatic glucose production (168). The impact of INSL-5 on glucose control in mice also appears dependent on the mode of glucose delivery, as blood glucose or insulin levels were similar in $Insl5^{-/-}$ mice compared to WT following an oral glucose test (164, 168). As oral but not intraperitoneal glucose administration stimulates the parasympathetic aspects of the gut-brain axis to centrally mediate hepatic glucose production (168), these findings suggest that INSL-5 may influence glucose homeostasis via direct actions on hepatocytes to influence hepatic gluconeogenesis. Studies on the insulinotropic action of INSL-5 have produced conflicting results (167, 171). As *Insl5* is not expressed in pancreatic islets (164, 168), any direct effects of endogenous INSL-5 on islets would appear to occur in an endocrine fashion. Circulating INSL-5 levels are estimated to be in the picomolar range (164, 172), which is several orders of magnitude lower than the EC_{50} of INSL-5 on RXRP4 (166) and the supraphysiological concentrations used in the majority of insulin secretion experiments may have contributed to the conflicting results.

CONCLUDING REMARKS

Although enteroendocrine cells make up only 1% of the epithelial cell population along the GI tract (9), the hormones they secrete in response to one's nutritional status have profound impacts on peripheral metabolism (**Figure 2**). We have provided an overview of the metabolic actions of some of these gut hormones, including their role in maintaining glucose homeostasis and energy balance. Under fasting conditions, ghrelin and INSL5 levels are elevated to induce hunger and to prevent hypoglycaemia. Conversely, during the postprandial period, elevated GIP and GLP-1 levels augment postprandial insulin secretion to prevent hyperglycaemia. In addition to its insulinotropic effect, GLP-1 also act in concert with PYY and OXM to induce satiety (**Figure 1**). Moreover, some of the impressive metabolic gains from bariatric surgeries have been ascribed to alterations in the secretory profile of gut hormones. Altogether, the enteroendocrine system represents an attractive therapeutic target for treating metabolic disease as the pleiotropic effects of different gut hormones can be exploited individually.

AUTHOR CONTRIBUTIONS

ES and AM wrote the manuscript. DK and RY critically reviewed the manuscript. All authors approved the final version for publication.

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Gut Mechanisms Linking Intestinal Sweet Sensing to Glycemic Control

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Sensing nutrients within the gastrointestinal tract engages the enteroendocrine cell system to signal within the mucosa, to intrinsic and extrinsic nerve pathways, and the circulation. This signaling provides powerful feedback from the intestine to slow the rate of gastric emptying, limit postprandial glycemic excursions, and induce satiation. This review focuses on the intestinal sensing of sweet stimuli (including low-calorie sweeteners), which engage similar G-protein-coupled receptors (GPCRs) to the sweet taste receptors (STRs) of the tongue. It explores the enteroendocrine cell signals deployed upon STR activation that act within and outside the gastrointestinal tract, with a focus on the role of this distinctive pathway in regulating glucose transport function via absorptive enterocytes, and the associated impact on postprandial glycemic responses in animals and humans. The emerging role of diet, including low-calorie sweeteners, in modulating the composition of the gut microbiome and how this may impact glycemic responses of the host, is also discussed, as is recent evidence of a causal role of diet-induced dysbiosis in influencing the gut-brain axis to alter gastric emptying and insulin release. Full knowledge of intestinal STR signaling in humans, and its capacity to engage host and/or microbiome mechanisms that modify glycemic control, holds the potential for improved prevention and management of type 2 diabetes.

Keywords: intestinal sweet taste receptors, L-cells, glucose transport, SGLT-1, glycemic control, type 2 diabetes mellitus

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INTRODUCTION

It is now widely recognized that the gastrointestinal tract is a major determinant of metabolic homeostasis, and the largest endocrine organ of the body. This is due to the diversity and wide signaling repertoire of the gastrointestinal enteroendocrine cells (EECs) which can, collectively, release over 30 different peptide hormones and neurotransmitters (1). To subserve this signaling function gastrointestinal EECs are configured either as "open" cells—possessing long, slim, finger-like extensions on their apical side to sense the luminal milieu and, in turn, release signaling molecules, or as "closed" cells which do not access the lumen, but can respond indirectly to luminal content (2). EEC have classically been sub-divided according to their hormone or transmitter content, and regional location within the gastrointestinal tract. However, the substantial overlap in transcriptional expression and subcellular stores that has recently been identified now supports a more heterogeneous EEC population (3, 4).

EECS RESPOND TO INTESTINAL CARBOHYDRATES

Exposure to luminal glucose generates signals that have a profound influence on intestinal motor and absorptive function. These signals include release of the gut peptides glucose-dependent insulinotropic polypeptide (GIP) from K-cells located in the proximal intestine and glucagon-like peptide-1 and 2 (GLP-1, GLP-2) from L-cells located in more distal regions of the intestine (5, 6), and release of the bioamine serotonin (5-HT) from enterochromaffin (EC) cells located throughout the gastrointestinal tract (7–9). GLP-1 and GIP, the "íncretin" peptide hormones, are degraded rapidly upon release by the ubiquitous enzyme dipeptidyl peptidase-IV and neutral endopeptidase, with <50% of secreted hormone entering the circulation. However, they powerfully augment glucose-dependent insulin release in response to an enteral glucose load (in comparison to an intravenous isoglycemic glucose load) (10, 11). GLP-1 and 5-HT also activate GLP-1 and 5-HT_3 receptors on intestinal vagus nerve endings as key signals in the "gut-brain axis," which, in turn, triggers vagal reflexes to slow the subsequent emptying of carbohydrate from the stomach, and induce satiation (12, 13). Accordingly, the release of GLP-1, GIP, and 5-HT is crucial to the regulation of postprandial glycemia. In contrast, GLP-2, which is co-released with GLP-1, is intestinotrophic and a potent signal to upregulate the expression and function of the primary intestinal glucose transporter, sodium-glucose cotransporter-1 (SGLT-1) (14).

SWEET TASTE MACHINERY

Lingual Sweet Taste

All known sweet tastants, including hexose sugars, D-amino acids, sweet proteins (such as monellin and thaumatin), and low-calorie sweeteners (LCS) are sensed by a single broadly-tuned sweet taste receptor (STR), comprised of a heterodimer of class C, G-protein coupled receptors, T1R2, and T1R3 (15). In lingual sweet taste cells, where sweet taste transduction has been most fully characterized, the interaction of sweet tastants with STRs initiates dissociation of the G-protein, gustducin, into Gα and Gβγ subunits and activation of phospholipase C β_2 ($PLC\beta_2$); intracellular Ca^{2+} is then released from inositol 1,4,5-trisphosphate-sensitive (IP3) stores, leading to opening of the melastatin type-5 transient receptor potential cation channel (TRPM5) to sodium influx [for review, see (16)]. Increases in intracellular Na^+ and Ca^{2+} then depolarize the basolateral membrane and, via 5-HT and ATP-dependent pathways, activate intermediary taste cells and chorda tympani and glossopharyngeal nerves that convey taste information centrally to the insular cortex [for review, see (17)] (18–20).

Intestinal Sweet Taste

STRs are well-described on subsets of EEC in the proximal small intestine, with evidence of STR-equipped K-cells, L-cells, and EC cells in humans (21, 22). STRs are also documented widely in metabolic tissues that sense and respond to carbohydrates, such as pancreatic β-cells, hepatocytes, adipocytes, and hypothalamic neurons [for review, see (23, 24)]. Expression of intestinal STR, like many GPCR, is of low magnitude, and optimally detected with high sensitivity SYBR-based PCR approaches rather than Taq-based PCR. Evidence in rodents, and in human cells and tissues, provides strong support that intestinal STRs function as upstream sensors linked to the release of GLP-1 from L-cells, and 5-HT from EC cells, and genetic deletion of T1R3, or pharmacological blockade of STRs with lactisole, decreases glucose and LCS-evoked GLP-1 and 5-HT release (21, 25–27). This is also true for GLP-2 release, which is STR-dependent in rodents (28, 29) and inhibited by the murine STR inhibitor, gurmarin (30).

Clinical studies have also reported acute effects of LCS to augment GLP-1 release in the presence of glucose and have shown a dose-dependent effect of lactisole to attenuate glucose-induced GLP-1 release in healthy subjects (31–34). Despite this, the balance of clinical evidence indicates that, at least in acute settings, LCS do not contribute substantially to the circulating pool of GLP-1 in humans (35–37).

Interplay Between STRs and SGLT-1 Can Regulate Glycemic Responses

Enterocytes account for around 90% of all intestinal epithelial cells and are polarized cells consisting of apical and basolateral membrane domains (38). These cells transport nutrients from the gut lumen to the circulation, and for glucose, apical SGLT-1 is the primary intestinal glucose transporter in both humans and animals. SGLT-1 is expressed primarily in the small intestine with highest density in the jejunum followed by the duodenum and then ileum (39, 40). SGLT-1 enables glucose absorption by co-transporting sodium along the electrochemical gradient established by the basolateral sodium-potassium ATPase (38, 41). Glucose then enters the systemic circulation via the facilitative monosaccharide transporter, GLUT2, located on the basolateral membrane of enterocytes; GLUT2 is bidirectional and capable of moving glucose in or out of enterocytes depending on glucose concentration gradients (38).

Importantly, transport of the monosaccharide substrates of SGLT-1 (e.g., glucose and galactose) triggers incretin hormone secretion (20), an action attenuated when SGLT-1 is pharmacologically inhibited with the competitive antagonist phlorizin, or absent through genetic deletion in rodents (42, 43). Our group provided the first evidence that SGLT-1 substrates, even if not metabolized (such as the glucose analog 3-O-methyl-glucose, 3-OMG), have the capacity to stimulate GLP-1 and GIP secretion in humans (44). We have also established that SGLT-1-based transport is critical for *ex vivo* release of GLP-1 in human ileum, while blocking SGLT-1 with phlorizin or replacing extracellular Na^+ with N-methyl-D-glucamine abolishes this response (26).

In animals, a wide range of sweet stimuli are capable of upregulating SGLT-1 expression and function, including LCS (45–48), indicating that SGLT-1 activity is modulated by an upstream and broadly tuned sweet taste sensor. Accordingly, STRs may have the capacity to stimulate gut hormone release both directly, and indirectly by augmenting SGLT-1 function.

The latter is evidenced in mice lacking T1R3 or α-gustducin, where SGLT-1 expression and function are not increased in response to dietary glucose or LCS supplementation as occurs in control mice (42). Moreover, the 3-fold increase in jejunal SGLT-1 expression following 4 days of sucralose gavage (100 mg, twice-daily) in control mice was absent in our mice lacking both T1R2 and T1R3 subunits of the STR (Marino Z, Young RL; **Figure 1**). Together, these experiments attest to the importance of intestinal STRs in regulating SGLT-1 function in mice, and support the notion that LCS can potentiate postprandial glycemic excursions via STR-dependent gains in SGLT-1 function and glucose absorption, in response to habitual consumption of sugars or LCS (**Figure 2**).

There is evidence that enteric neurons link glucose sensing in EEC to glucose transport function in enterocytes (50). Studies in rodents have shown that intestinal areas adjacent to regions exposed to LCS have increased SGLT-1 expression (46). This communication between STR-equipped L cells and SGLT-1-bearing absorptive enterocytes is likely to involve gut hormone intermediaries, such as GLP-1 and/or GLP-2. Indeed, GLP-2 receptors are present on enteric neurons in guinea pig ileum, mouse jejunum, mouse and pig intestine (20, 51, 52) and absorptive enterocyte progenitors in mouse jejunum respond to GLP-2 in an enteric neuron-dependent manner (52). GLP-2 is also capable of upregulating SGLT-1 expression *in vivo* (28), and STR-dependent release of both GLP-1 and GLP-2 is detected at higher concentrations in the portal and lymphatic circulation than the systemic circulation in rodents (28, 53). This indicates that local release of either mediator in response to sweet stimuli, including LCS, may be sufficient to increase SGLT-1 function. It may also, in part, explain the equivocal nature of human data on LCS-evoked gut hormone release, as paracrine signaling in the mucosa could occur in the absence of a substantial contribution to circulating hormone levels. To this end, we provided the first evidence that LCS evoke *ex vivo* GLP-1 release from human ileal tissue (26). However, the precise signal transduction pathways utilized by LCS to trigger gut hormone release in human mucosa remain to be identified.

An increase in SGLT-1 protein in the apical brush border of enterocytes occurs in a cyclic AMP (cAMP)-dependent manner in response to transduction of basolateral signals (54, 55), and secondary to an increase in SGLT-1 transcription (56) and stabilization (increased half-life) of the 3′-untranslated region of the SGLT-1 transcript (57, 58). This facilitates an increase in apical SGLT-1 protein translation and insertion in response to gut hormone signaling. Jugular vein infusion of GLP-2 increases the abundance of SGLT-1 protein and rate of SGLT-1-dependent glucose transport in the apical membrane of jejunal enterocytes in rats, a response abolished when protein translocation is inhibited with brefeldin (29, 59). This highlights the importance of GLP-2 in the regulation of SGLT-1 function at the apical brush border membrane.

While enteric neurons express receptors for other gut hormones, including GIP, GIP is unlikely to be responsible for glucose or LCS effects on SGLT-1 (20, 60). GIP receptor knockout and wild type mice show similar increases in jejunal SGLT-1 expression on a high carbohydrate diet, compared to mice on a low carbohydrate diet (20). Irrespective of which mucosal mediator is a trigger upon intestinal STR activation, the interplay between these broadly-tuned receptors and SGLT-1 is critical for glucose absorption and represents a major mechanism regulating overall glycemic control.

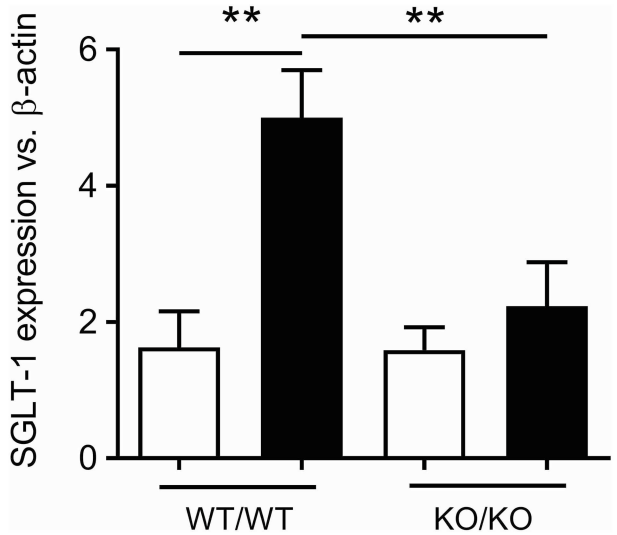

FIGURE 1 | STR-dependence of SGLT-1 expression in mice. Increased jejunal expression of SGLT-1 mRNA in 10 week-old control (WT/WT) mice gavaged for 4 days with sucralose (black bars) compared to water (white bars), and to mice homozygous for both *Tas1r2* and *Tas1r3* genes (KO/KO). Breeding pairs of mice homozygous for the *Tas1r2* or *Tas1r3* gene (129X1/SvJ mice backcrossed for at least 3 generations with C57BL/6 mice) were provided by Prof Charles Zuker (University of California, San Diego, USA). Mice homogenous for each gene were then paired to produce mice heterozygous for *Tas1r2* and *Tas1r3*. These mice, in turn, were paired to generate mice heterozygous, homozygous, and wild-type for both genes. From these mice, double homozygous (KO/KO) and wild-type littermate controls (WT/WT) were the subject of gavage experiments. Ten-week old male mice ($N = 5$ per group) maintained under standard housing and diet conditions in the SA Pathology Animal Care Facility were gavaged twice daily with 100 mg sucralose (Redox Chemicals, Minto, NSW Australia) in 200 μL water, or 200 μL water, at 0800 and 1800 over 4 days. These mice were fasted overnight then humanely killed at 0800, total RNA extracted from the jejunal mucosa, and real-time RT-PCR performed using primer assays for SGLT-1 (QT00112679) and β-actin (QT01136772, Qiagen, Sydney, NSW Australia) relative to expression of β-actin, as described (49); SGLT-1 expression was compared between groups and gavage regime by analysis of variance (ANOVA), adjusted for multiple comparisons by Holm-Sidak's correction (GraphPad Prism 7.02, San Diego, CA, USA). This experiment was approved and performed in accordance with guidelines of the Animal Ethics Committees of The University of Adelaide and SA Pathology (Adelaide, Australia). Data is shown as Mean ± SEM; ** $P <$ 0.01. We thank Prof Charles Zuker for generously supplying the homozygous *Tas1r2* and *Tas1r3* mice.

TYPE 2 DIABETES IS ASSOCIATED WITH STR DYSREGULATION

Globally, over 400 million people are living with diabetes, projected to rise to over 600 million by 2040 (61). Effective control of glycemia, as assessed by glycated hemoglobin (HbA1c) <6.5–7.0% (48–53 mmol/mol), is important to minimize the risk of the development and progression of microvascular

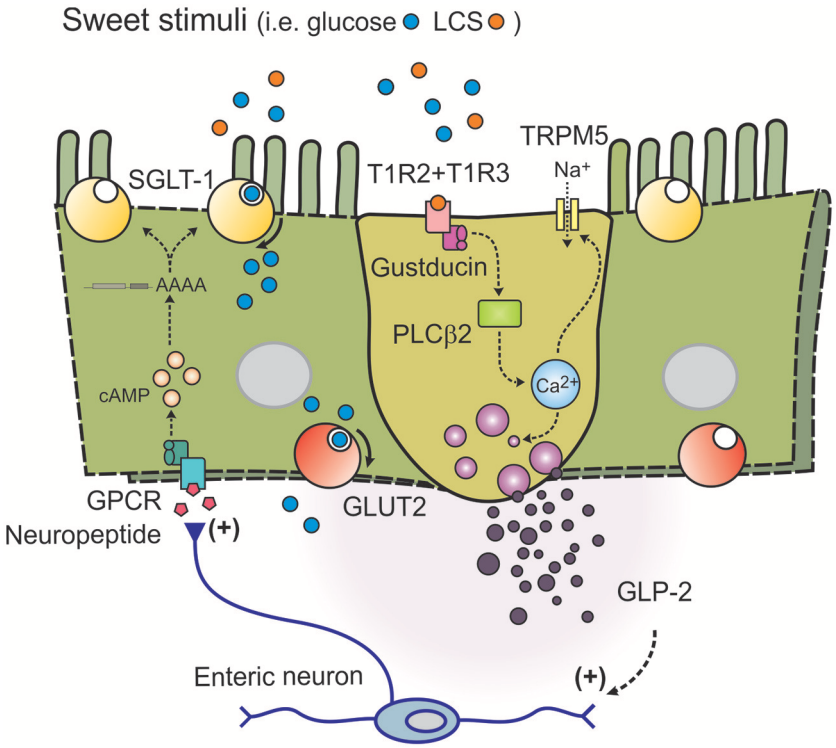

FIGURE 2 | Model of intestinal sweet taste sensing and signaling effectors. Sweet stimuli, including LCS, bind to STR comprised of a heterodimer of G-protein coupled receptors T1R2 and T1R3. Upon receptor binding an intracellular signaling cascade is activated, initiated by dissociation of G-protein gustducin into Gα and Gβγ subunits and activation of phospholipase C β_2 (PLCβ_2); intracellular Ca^{2+} is then released from inositol 1,4,5-trisphosphate-sensitive stores, leading to opening of the melastatin type-5 transient receptor potential cation channel (TRPM5) to sodium influx. Increases in intracellular Na^{+} and Ca^{2+} then depolarize the basolateral membrane, to facilitate release of peptide hormones such as GLP-2. GLP-2 may then trigger an enteric neuron pathway to release an unknown neuropeptide at nearby absorptive enterocytes leading to adenylate cyclase-dependent stablilization of the 3' end of SGLT-1 mRNA (to increase half-life), and SGLT-1 translation and insertion into the apical brush border membrane.

complications (i.e., eye, kidney, and nerve damage), and to a lesser extent, macrovascular complications. In the majority of patients with type 2 diabetes, who are relatively well-controlled, postprandial glycaemic excursions predominate over fasting blood glucose levels in contributing to HbA1c (62), and are determined by meal composition, the rate of gastric emptying, hepatic and peripheral glucose metabolism, intestinal glucose absorption, and insulin secretion and resistance (63). Meal-related secretion of insulin is augmented through the insulinotropic actions of the incretin hormones GIP and GLP-1 to reduce postprandial glycemic excursions in health (64, 65); in type 2 diabetes, a markedly attenuated insulinotropic action of GIP (66) and, in some cases, attenuated secretion of GLP-1 (67), contribute to an impairment of postprandial insulin secretion, so that the latter is insufficient to maintain euglycaemia. Furthermore, gut-derived 5-HT can also modulate glucose and energy homeostasis (68–70), and is augmented in patients with type 2 diabetes (71) and the obese (9).

The recognition that the gut, and EEC signals, are major determinants of glycemic control is attested to by the successful deployment of incretin-based therapies for type 2 diabetes. These include mimetics of GLP-1, GLP-1/GIP dual receptor agonists, and inhibitors of dipeptidyl peptidase-IV, which inactivates endogenous GLP-1 (72). These pharmaceutical compounds have improved clinical management of type 2 diabetes substantially, but their use is compromised by cost, compliance with administration, adverse gastrointestinal effects, or suboptimal efficacy in some patients.

While experiments in animal models and patients with type 2 diabetes have shown a gain in function of SGLT-1 and corresponding increase in the rate of intestinal glucose absorption (73, 74), the targeting of intestinal glucose absorption has received comparatively little attention. Indeed, it is likely that a proportion of the clinical benefits of the anti-diabetic gliflozin-class agents (SGLT-2 inhibitors) are due to actions at intestinal SGLT-1. This is particularly true for first-in-class examples, such as the dual SGLT-1/SGLT-2 inhibitor sotagliflozin, which has lower selectivity for SGLT-2 and acts beyond inhibition of renal glucose reabsorption by SGLT-2 to induce partial inhibition of intestinal SGLT-1, leading to augmented GLP-1 and insulin secretion, and a reduction in postprandial glucose excursions (75).

To assess whether regulation of intestinal STR was disrupted in patients with type 2 diabetes, and had an unfavorable impact on glucose absorption and postprandial hyperglycemia, we compared intestinal STR expression in individuals with and

without type 2 diabetes. We first established that STRs were expressed at similar levels in the duodenum in both groups when sampled at euglycemia (49). However, we found that T1R2 expression was decreased following enteral glucose exposure under hyperglycemic conditions in non-diabetic subjects, but remained elevated in patients with type 2 diabetes, where it was linked to an increase in glucose absorption (assessed by serum levels of 3-OMG which had been co-administered with the glucose load) (22). These findings support the notion that intestinal STR dysregulation in type 2 diabetes can exacerbate postprandial glycemic excursions. Furthermore, given that patients with type 2 diabetes are 3-fold more likely to consume beverages sweetened with LCS than healthy individuals (76), it is possible that high dietary LCS consumption contributes to, rather than alleviates, postprandial glycemic dysregulation.

LOW-CALORIE SWEETENERS AND GLYCEMIC CONTROL

Sugar-sweetened beverages contain high levels of sucrose or high fructose corn syrup (77) and represent a major source of added sugars in western diets. They account for around 16% of daily caloric intake of adults in the United States (78) and 11% in Canada and Australia (79), a level that exceeds the World Health Organization recommendation that added sugar consumption should be limited to 10% of daily caloric intake (80). These sugars are rapidly absorbed by the small intestine to increase glycemic load, which, when associated with increased peripheral insulin resistance, increases the risk of developing type 2 diabetes (81).

The outcomes of epidemiological studies indicate that high and habitual consumption of sugar-sweetened beverages is associated with an increased risk of developing type 2 diabetes, independent of total energy intake or body mass (77, 82). While these findings do not establish causality (83, 84), the adverse health outcomes linked to high sugar consumption have led to changes in global health policy to limit such intake, with several countries now implementing a sugar tax (80, 85). Not surprisingly, beverages sweetened with LCS have become a popular alternative.

Diet beverages contain a single LCS, or more frequently, LCS combinations, in place of sugars (86), with specific LCS commonly identified by their European Food Safety Authority E-number, i.e., aspartame (E951), sucralose (E955), and acesulfame-K (E950). LCS differ substantially in their oral bioavailability and, therein, exposure to intestinal regions and their microbiota. For example, aspartame is completely hydrolyzed in the proximal intestine to methanol and constituent amino acids, aspartate and phenylalanine, and has no effective oral bioavailability or exposure to the distal intestine and its microbiota. Sucralose has low oral bioavailability (around 15%), but full exposure to the intestine and microbiota due to excretion in largely unchanged form in feces; minor absorbed sucralose and glucoronidation end-products undergo renal excretion. Finally, acesulfame-K has high oral bioavailability (90–100%) due to rapid absorption in the proximal intestine and has limited exposure to the distal intestine and its microbiota; acesulfame-K is cleared via renal excretion in largely unchanged form [for reviews, see (87–89)]. These distinct properties should be considered in interpreting effects of LCS both within, and outside, the gastrointestinal tract.

LCS are 200 to 13,000 times sweeter than sucrose by weight, and were expected to be beneficial in the setting of obesity and type 2 diabetes due to their low calorie content. There is, however, only equivocal evidence of this benefit, with several epidemiological studies indicating little or no benefit, or even an increased risk of weight gain (90–92). Moreover, some epidemiological studies suggest that a high habitual intake of beverages sweetened with LCS is associated with an increased risk of developing type 2 diabetes (93–97). Reverse causality (e.g., people opting for LCS-sweetened beverages in response to weight gain and/or obesity, or subclinical disease including pre-diabetes) is unlikely to fully account for the increased risk, which is evident even after adjusting for differences in body mass and energy intake. Furthermore, two studies have reported an elevated risk of developing type 2 diabetes in normal weight individuals (93, 97).

The outcomes of studies that have prospectively investigated the effects of LCS intake on long-term glycaemic control (assessed by HbA1c) or insulin resistance have been equivocal, and several failed to adjust for differences in sugar intake (76, 98–102). Despite this, high habitual patterns of LCS consumption have been reported to increase HbA1c levels in healthy adults, independent of body mass (101), while daily LCS consumption has been dose-dependently associated with HbA1c increases in type 2 diabetes (76). A negative impact of LCS on acute glycemic control has also been shown in obese individuals, where a sucralose preload consumed in advance of an oral glucose tolerance test augmented blood glucose levels over the following 5 h substantially, when compared to water or no preload (103).

Collectively, the potential for LCS to impair glycemic control remains uncertain, in large part due to the small number of prospective clinical studies (104, 105). Proposed mechanisms linking LCS to an increased risk of developing type 2 diabetes in humans include a reduced fidelity of central responses to nutritive stimuli, effects on gut microbiota, and an effect of LCS to augment glucose absorption.

We recently reported early findings of a randomized placebo-controlled clinical study investigating the effect of diet supplementation with combined LCS (sucralose 276 mg, acesulfame-K 156 mg in capsules; equivalent to 1.5 L diet beverage/day) over 2 weeks on glycemic responses to enteral glucose. We observed a clinically significant effect of LCS to increase the rate of glucose absorption and augment blood glucose responses to enteral glucose in healthy subjects consuming LCS, relative to placebo. Moreover, glucose-evoked GLP-1 and GLP-2 release was decreased in LCS-consuming participants, which may relate to the more rapid proximal absorption of glucose limiting the exposure of more distally located L-cells (106). These findings indicate a negative impact of habitual high LCS intake on glucose absorption and acute glycaemic control in health, and add support for the concept that high habitual intake of LCS may increase the magnitude of postprandial glycemic excursions.

LCS AND THE GUT MICROBIOME

The gut microbiome comprises the diverse range of bacteria, yeasts, and other microorganisms which exist in a largely symbiotic relationship with the host (107). These prevent potentially harmful microorganisms from colonizing the gut by competing for energy resources (108). Use of these resources liberates nutrients which would be otherwise inaccessible to the host, i.e., microbial conversion of indigestible polysaccharides to short chain fatty acids (SCFAs) such as acetate, propionate, and butyrate, which act as substrates for cellular metabolism, gluconeogenesis and lipogenesis. Moreover, SCFAs play a crucial role in satiety signaling, and modulate appetite directly and indirectly via leptin synthesis in adipose tissue (109). SCFAs also have a beneficial impact on glycemia, with propionate shown to improve insulin sensitivity, and butyrate to prevent or improve insulin resistance in mice fed a high fat diet (110–112). Microbial-derived signals from the gut, therefore, have the potential to influence glycemic control substantially.

The composition of the gut microbiome of individuals with type 2 diabetes differs from that of non-diabetic individuals, in its relative and BMI-independent decrease in abundance of species from the *Clostridium* phylum (113, 114). These species are negatively correlated with markers of poor glycemic control such as fasting glucose, HbA1c and insulin, but positively correlated with the insulin sensitizing hormone adiponectin (113). Alterations in the gut microbiome of individuals with type 2 diabetes are also associated with changes in functional microbial genes, with a specific enrichment of pathways for starch, glucose, fructose, and mannose metabolism, which increases the potential for energy harvest and metabolism (113). These changes are causally related to the development of insulin insensitivity and resistance, as allogenic transplantation of intestinal microbiota from lean donors to recipients with the metabolic syndrome improved insulin sensitivity (115). This highlights the importance of the gut microbiome composition with respect to the development of metabolic disorders, including type 2 diabetes.

Exposure to LCS has been shown to drive glucose intolerance in mice via a LCS-dependent shift in composition of the gut microbiome ("dysbiosis"). Transplantation of fecal microbiota from donor mice supplemented chronically with LCS (saccharin)

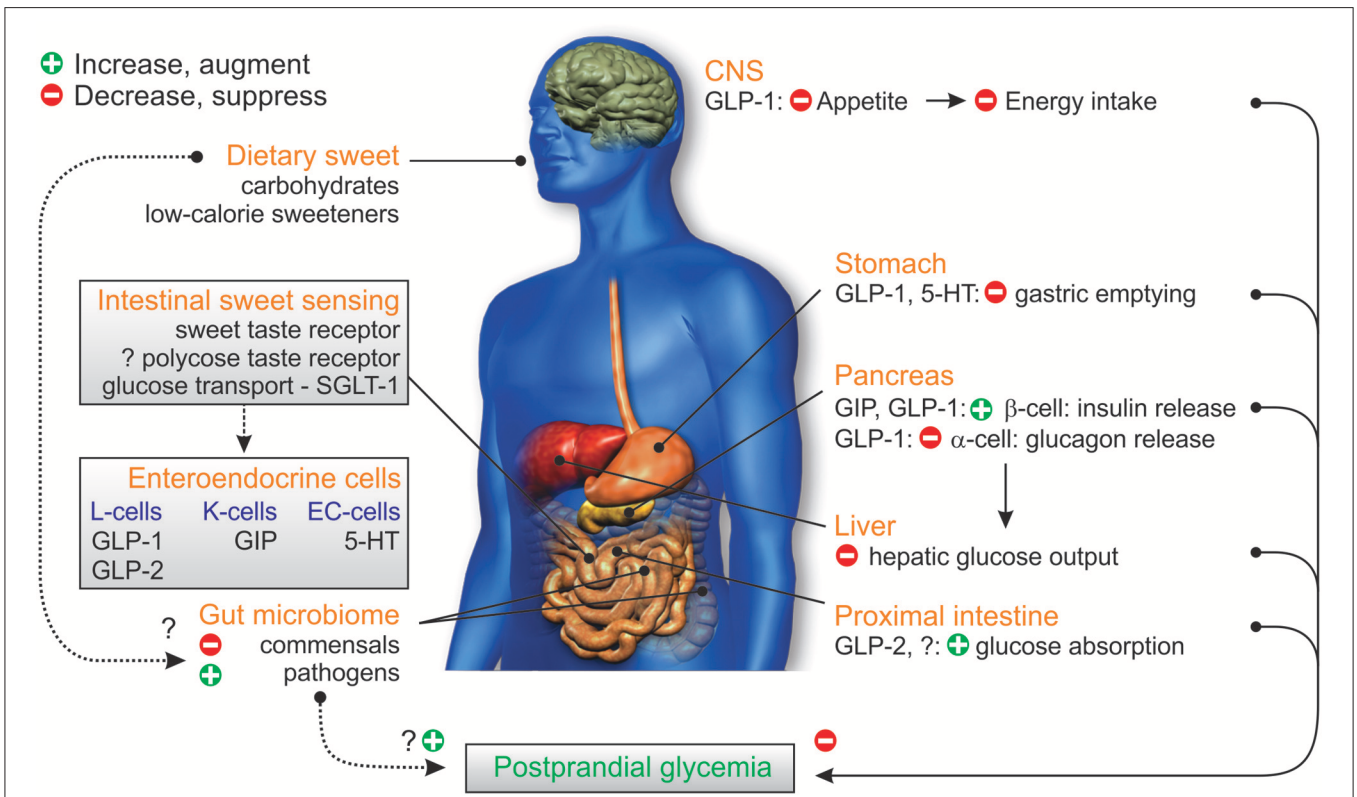

FIGURE 3 | Gastrointestinal factors influencing glycemic control. Dietary sweet stimuli can activate STR in the proximal intestine facilitating the enteroendocrine cell release of the incretin peptides GIP from K-cells and GLP-1 from L-cells, as well as 5-HT from EC-cells; substrates of SGLT-1 (glucose, galactose) also trigger GIP and GLP-2 release. GIP and GLP-1 stimulate glucose-dependent insulin release, to increase glucose disposal; GLP-1 and 5-HT also slow the rate of gastric emptying via vagus nerve signals (not shown) while GLP-1 inhibits pancreatic glucagon release, leading to reduced hepatic glucose output. GLP-2 co-released from L-cells acts to increase intestinal glucose absorption via an increase in the capacity for SGLT-1-based glucose transport. Dietary sweet stimuli can also alter the composition of the gut microbiome in favor of colonization of gut pathogens over fermentative gut commensals, which can affect energy harvest, and disrupt microbiome signaling to the host and glycemic control. Together these influences can disrupt the homeostatic balance between glucose-evoked gut hormone release, glucose absorption, and microbiome composition, leading to dysglycemia which would potentially be harmful in the setting of type 2 diabetes. In addition, complex carbohydrates (oligosaccharides) may contribute to these processes via a yet to be identified polycose taste receptor.

to germ-free recipient mice resulted in glucose intolerance after 6 days. Changes in abundance of more than 40 operational taxonomic units were demonstrated in the recipient mice, along with an upregulation of microbial carbohydrate-related metabolic pathways, and an increase in fecal SCFA levels (101). This increase in SCFAs was speculated to represent increased microbial energy harvest, but may equally represent the outcome of differences in intestinal transit time or absorption (116, 117). It is also been unclear whether fecal bacterial samples accurately represent the microbiome of the proximal gut (118). Indeed, Daly et al. showed that supplementation with SUCRAM (neohesperidin dihydrochalcone and saccharin) over 2 weeks in weaned piglets increased the abundance of *Lactobacillaceae* in cecal, but not fecal, samples, while cecal SCFA levels were comparable in the LCS and control diet groups (117). These findings underscore the importance of testing regional (or mucosa-associated) bacteria in the gut, and of establishing causal mechanisms as opposed to microbial followers of changes in host metabolism.

Causal mechanisms linking dysbiosis to impaired GLP-1 signaling in the gut-brain axis were recently investigated in mouse models of diet-induced type 2 diabetes. Grasset et al., identified a subset of ileal bacteria in these mice that disrupted GLP-1-dependent nitric oxide production in ileal enteric neurons via an attenuation of GLP-1 receptor expression, and showed that this drove GLP-1 resistance in the regulation of gastric emptying and insulin release (119). A GLP-1 resistant phenotype in germ-free mice was rescued through conventionalization with ileal bacteria from control-fed mice, but not from mice fed the diabetogenic diet, while antibiotic treatment led to GLP-1 resistance in control-fed mice, but improved GLP-1 resistance in diabetogenic diet-fed mice. This study demonstrated that diabetogenic diet-induced gut dysbiosis was causally related to dysglycemia via disruption of GLP-1 signaling in the gut-brain axis, but did not extend to an assessment of specific bacterial populations or products that mediated this effect.

Accordingly, clinical studies are now required to determine whether LCS induce intestinal dysbiosis in humans, whether this is causally related to disruption of the gut-brain axis that controls glycemia, and which microbiome-derived signals effect this change. Such investigation holds the potential to usher in new classes of anti-diabetic therapy which would correct defects in microbiome composition and/or associated signaling pathways that impact glycemic control adversely.

TASTING SWEET VIA NON-STR PATHWAYS

Several studies have reported the existence of a lingual and STR-independent sensor tuned to detect the nutritive value of complex carbohydrates. Behavioral studies in rodents have shown that rats prefer consumption of polycose (glucose oligomer) solutions above that of water or solutions of the disaccharides sucrose and maltose, particularly at low concentrations (120, 121). This was further supported by electrophysiology studies of lingual nerve activity, which indicated that rats could distinguish the tastes of polycose and sucrose (122, 123). Importantly, mice lacking one or both STR subunits had limited, or no behavioral or lingual nerve responses to simple sugars, while responses to polycose remained normal (124–127). More recently, behavioral research on human taste detection have added support for a human polycose taste receptor, showing that humans can detect glucose oligomer solutions on an equimolar basis to simple sugars, even when lingual STR were blocked with lactisole and amylase activity inhibited by an α-glucosidase inhibitor (to prevent oral breakdown of glucose oligomers to STR-detectable mono- and disaccharides) (128–130). The latter study also indicated that oligosaccharides of 4 or higher degrees of polymerization (i.e., maltotetraose) were detected by a STR-independent lingual taste pathway in humans. While a polycose receptor is yet to be cloned, future characterization may also reveal its potential as an intestinal nutrient sensor, and whether there are associated consequences for glycemic control in humans.

CONCLUSION

Although foods and beverages sweetened with LCS have become a popular alternative to their sugar-sweetened counterparts, research relating to their impact on acute and chronic human health has been inappropriately limited, and the outcomes equivocal. However, the outcomes of the hitherto small number of well-conducted studies raises concerns regarding their health impact. Further research is now required to better characterize the EEC biology of intestinal sweet taste signaling in humans, characterize the mechanisms utilized by LCS to impact glycemic control, and identify potential targets capable of modifying STR signaling for clinical benefits (**Figure 3**). In addition, studies are needed to determine whether patterns of LCS consumption can trigger gut dysbiosis, with consequences for human health as are subsequent metagenomic, metabolomic, and functional investigations of causal mechanisms. These hold the high potential for improved prevention and novel management of type 2 diabetes.

AUTHOR CONTRIBUTIONS

DK, DJK, TW, MH, CR, and RY were all involved in conception, design, and writing of the manuscript. All authors have approved the publication of this final version of the manuscript.

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REVIEW
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Pleiotropic Effects of GLP-1 and Analogs on Cell Signaling, Metabolism, and Function

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The incretin hormone Glucagon-Like Peptide-1 (GLP-1) is best known for its "incretin effect" in restoring glucose homeostasis in diabetics, however, it is now apparent that it has a broader range of physiological effects in the body. Both *in vitro* and *in vivo* studies have demonstrated that GLP-1 mimetics alleviate endoplasmic reticulum stress, regulate autophagy, promote metabolic reprogramming, stimulate anti-inflammatory signaling, alter gene expression, and influence neuroprotective pathways. A substantial body of evidence has accumulated with respect to how GLP-1 and its analogs act to restore and maintain normal cellular functions. These findings have prompted several clinical trials which have reported GLP-1 analogs improve cardiac function, restore lung function and reduce mortality in patients with obstructive lung disease, influence blood pressure and lipid storage, and even prevent synaptic loss and neurodegeneration. Mechanistically, GLP-1 elicits its effects via acute elevation in cAMP levels, and subsequent protein kinase(s) activation, pathways well-defined in pancreatic β-cells which stimulate insulin secretion in conjunction with elevated Ca^{2+} and ATP. More recently, new studies have shed light on additional downstream pathways stimulated by chronic GLP-1 exposure, findings which have direct relevance to our understanding of the potential therapeutic effects of longer lasting analogs recently developed for clinical use. In this review, we provide a comprehensive description of the diverse roles for GLP-1 across multiple tissues, describe downstream pathways stimulated by acute and chronic exposure, and discuss novel pleiotropic applications of GLP-1 mimetics in the treatment of human disease.

Keywords: GLP-1, signaling, diabetes, metabolism, cell function and integrity

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INTRODUCTION

While its gene was first cloned in 1983, and protein product approved as a therapeutic agent for Type 2 diabetes mellitus (T2D) in 2005, the mammalian glucagon-like peptide-1 (GLP-1), its modes of action, and various analogs, have been and are still widely studied. As it is a highly attractive T2D therapy, the major known functions of the incretin peptide GLP-1 and analogs are based on studies delineating its role in the endocrine pancreas. GLP-1 acts through binding to its receptor (GLP-1R), triggering a downstream signaling cascade able to induce a potent stimulation of glucose stimulated insulin secretion (GSIS) in β-cells, as well as inhibition of α-cell glucagon release. GLP-1 analogs, such as Liraglutide and Exendin-4, unlike endogenously produced GLP-1, are not rapidly degraded by Dipeptidyl peptidase-4 (DPP-4) and, therefore, can induce sustained therapeutic actions, that

otherwise would not be possible due to the exceedingly short half-life of endogenous GLP-1 in circulation. GLP-1R is a B class G-protein-coupled receptor abundantly expressed in the pancreas and central nervous system, but also detected in lower levels in the gut, kidneys, lungs, liver, heart, muscle, peripheral nervous system, and other tissues (1). Upon binding to the receptor, GLP-1 and its analogs also initiate a variety of additional anti-diabetic effects, including, but not limited to, reduction in gastric emptying, increase in satiety and inhibition of food motivated behavior, replenishment of insulin stores, as well as cytoprotective and anti-inflammatory actions on β-cells (2–9). To initiate these beneficial effects, however, GLP-1 must first be secreted from either the enteroendrocine L-cells, the preproglucagon (PPG) neurons located in the nucleus of the solitary tract of the brain stem, or, as reported recently, the α-cells in the pancreas (10–14). Upon ligand binding, GLP-1R initiates a cascade that involves activation of membrane bound Adenyl Cyclase (AC) and consequent production of cyclic adenosine monophosphate (cAMP). Downstream of cAMP formation, several signal transduction pathways can be initiated, which generally require activation of either one or both of the cellular cAMP effectors, Protein kinase A (PKA) and exchange protein directly activated by cAMP (EPAC) [reviewed in (15, 16)].

GLP-1R mediated effects arise as a consequence of the immediate signaling cascade, which can impact insulin secretion and calcium flux in a rapid post translational modifications based manner (4, 17), and/or, the late stage or chronic effects, which can operate through modulation of gene expression and cellular metabolism (18–21). To date, the vast majority of studies have tended to focus on the acute impact of GLP-1R activation. More recently, research has begun elucidating the consequences of chronic GLP-1R stimulation (19, 22–25). Long-lasting GLP-1 analog treatments are now in regular clinical use, and their impact, safety and efficacy are well-established and extensively reviewed (25–31). However, a succinctly summarized and current understanding of the signaling mechanisms and metabolic impact of chronic GLP-1R agonist activity on β-cells, and more broadly, across other tissues is both essential and lacking. To this end, our review outlines the current knowledge in regards to GLP-1R activation, subsequent signaling events, and discuss recent findings, firstly with respect to the well-characterized pancreatic β-cell, followed by effects on other cell and tissue types.

ACUTE EFFECTS OF GLP-1 IN β-CELLS

Glucose enters the pancreatic β-cells via the transporter, *Glucose transporter 2* (GLUT2), moving down a concentration gradient from the capillaries. In the cytosol glucose is phosphorylated by the enzymes glucokinase/hexokinase (glucokinase is the predominant isoform in the β-cell), after which it enters the glycolytic pathway. Rapid catabolism of glucose via glycolysis and mitochondrial TCA cycle activity generates ATP (32, 33). The subsequent increase in ATP/ADP ratio leads to a closure of ATP-sensitive K^+ channels, intracellular accumulation of K^+ ions and subsequent membrane depolarization, causing an influx of Ca^{2+} via voltage dependent Ca^{2+} channels (VDCC). This Ca^{2+} influx, along with elevated ATP, results in exocytosis of the plasma membrane docked immediate release pool (IRP) of insulin granules, a sub-pool of the readily releasable pool (RRP) which contains ∼1–5% of available insulin granules (16, 34). This is the main driver behind β-cell 1st phase stimulus-secretion coupling, since it is the products of glucose catabolism that ultimately drive insulin exocytosis. This release is rapid, and is known to peak at around 10 min from the initial glucose challenge, whilst the second phase of insulin release, which is sustained, consists in the release of granules from the larger Reserve pool (RP), containing ∼95–99% of insulin granules, and lasts until glucose stimulation ends (30–60 min under normal physiologic conditions) (16, 35). Before the trafficking and release of the RP granules occur, granule competency must be achieved, and this is believed to occur through granule acidification resultant from an increase of H^+ and Cl^- ions and processing of pro-insulin into mature, releasable, insulin (36).

In pancreatic β-cells, GLP-1R stimulated pathways act promptly (seconds to minutes) to potentiate glucose-dependent insulin release. This is achieved by a rapid increase in cAMP, which is accompanied by direct activation of PKA and EPAC. These two effectors of cAMP signaling modify several targets within the secretory machinery, with the net effect to synergistically enhance the amount of insulin secreted in response to glucose stimulation (15, 16). Indeed, several independent mechanisms are also reported to act in concert in order to result in enhanced insulin secretion, as discussed below **(Figure 1).**

Activation of PKA by cAMP results in release of its two catalytic subunits from the two anchoring regulatory subunits from specific cellular locations and anchoring proteins. Activated PKA can directly phosphorylate the sulphonylurea receptor (SUR1 as well as a regulatory subunit of K^+ATP channels, thereby reducing SUR1 affinity to ADP, and increasing activity of $K_{ir6.2}$, respectively (37). This, in turn, leads to channel closure and increased accumulation of intracellular K^+ ions (9), influx of Ca^{2+} and promotion of insulin secretion in response to GLP-1 stimulation.

Another cAMP effector, EPAC, is implicated in K^+ATP channel regulation. Kang, et al. demonstrated that activation of EPAC reduces the concentration of ATP required to achieve closure of K^+ ATP channels (38). This indicates that in the presence of active EPAC, lower concentrations of ATP promote membrane depolarization and subsequent insulin granule exocytosis. Indeed, acute exposure to EPAC can stimulate insulin granule exocytosis and maturation, through sensitization of the ryanodine receptors and activation of the calcium sensing complex (16, 39, 40). EPAC aids insulin priming and release via facilitating formation of a Rim2/Rab3a complex via Rim2/EPAC interaction (3, 41–43). Rim2/Rab3a complex interacts with the Ca^{2+} sensor Piccolo-CAZ (cytoskeletal matrix protein that associates with the active zone) to facilitate vesicle exocytosis at the cytoplasmic surface of the insulin granule (3, 41, 42, 44). However, enhanced vesicle mobilization, priming, and subsequent exocytosis is not only regulated by the EPAC pathway, but also directly by PKA. PKA can facilitate insulin secretion

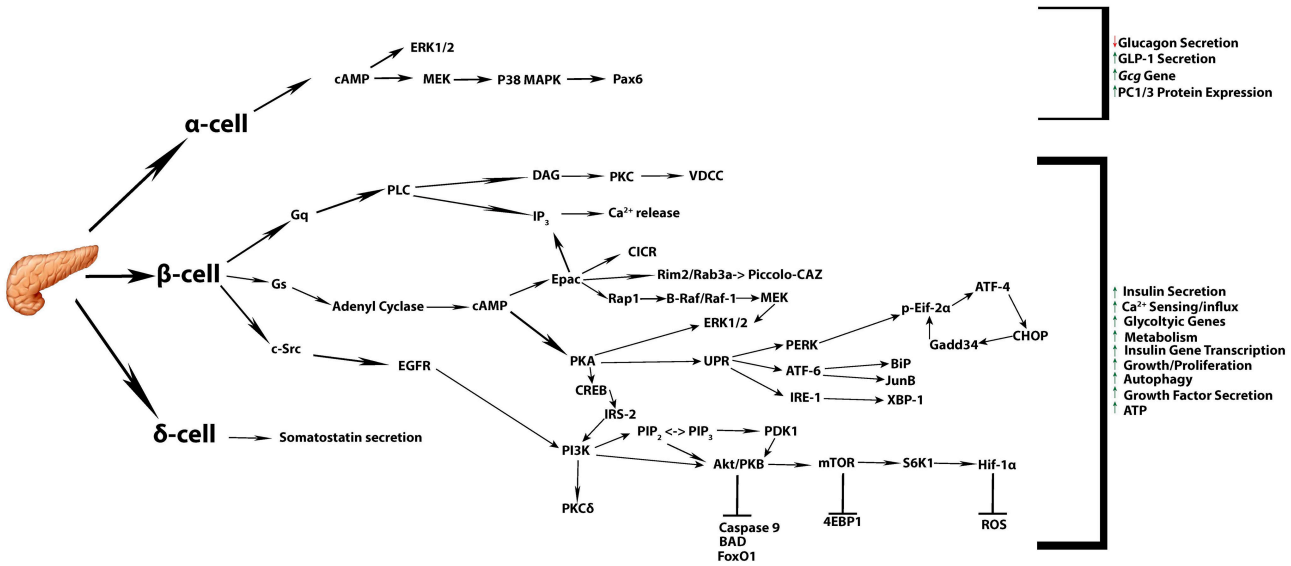

FIGURE 1 | Commonly accepted GLP-1 signaling in the pancreatic tissue. Summary of the most commonly known signaling cascades activated by GLP-1 in the three different endocrine cell types α, β, δ, and their overall impact on diverse cellular processes.

through regulation of Ca^{2+} secretion, whereby PKA sensitizes the inositol triphosphate receptor leading to release of Ca^{2+} from intracellular stores (16, 45–47). PKA has also been reported to accelerate the competency and mobilization of vesicles from the reserve pool of insulin to the readily releasable pool and, thus, enhance Ca^{2+}-dependent exocytosis in mouse pancreatic islets (16, 34, 48, 49). Indeed, a recent report described that PKA activity is required for glutamate uptake into the insulin granules, with glutamate uptake potentiating insulin release (50). Cytoplasmic glutamate can be derived via the malate-aspartate shuttle following pyruvate mitochondrial metabolism. Since glucose metabolism is absolutely required for GLP-1-induced stimulation of insulin secretion, the latter mechanism represents a clear link between glucose metabolism and GLP-1 action via PKA to amplify insulin secretion.

CHRONIC EFFECTS OF GLP-1 IN β-CELLS

It is perhaps unsurprising that GLP-1 therapeutics show greater efficacy compared with traditional diabetic medicines, due to their potential to address not only acute stimulation of insulin secretion in response to a rise in blood glucose, but also slow the progressive loss of β-cell function and tissue mass in T2D. The beneficial effects of GLP-1 are resultant from cAMP mediated signaling, and ultimately activation of pro-survival cAMP responsive element binding (CREB) signaling, as well as the non-receptor tyrosine kinase/c-Src, transactivation of EGFR (5, 40, 51). CREB and EGFR pathways induce pro-survival and anti-apoptotic responses (52–57), including increased expression of anti-apoptotic genes (58), attenuation of ER stress (59), prevention of oxidative stress and fatty acid mediated toxicity (60). cAMP binds to PKA regulatory subunits, releasing and activating PKA catalytic subunits that cause phosphorylation of CREB at Ser^{133}, promoting its activation and subsequent binding to genes containing palindromic CRE repeat sequences. Activated CREB regulates the expression of several genes essential for normal β-cell function, including the insulin gene (61). Additionally, EPAC can also exert late stage effects through the Rap1 protein, a small GTPase that regulates B-Raf/Raf-1 activation via a combination of residue phosphorylation (Ser^{338}) and dephosphorylation (Ser^{259}), which enables Raf to phosphorylate the mitogen-activated protein kinase (MEK). MEK, in turn, phosphorylates the threonine and tyrosine residues of the extracellular-signal regulated kinases (ERK) 1 and 2, which regulate gene expression, growth and differentiation (62, 63). In addition to protection against apoptosis, it has been proposed that GLP-1 induces β-cell proliferation in rodent cell lines and in isolated rodent islet cells (54, 55, 64). However, these findings have varying results in human cells, with a recent study identifying an age-dependent Exendin-4 induced signaling mechanism regulating β-cell proliferation (65). This study revealed that unlike adult human islets, juvenile human islets transplanted into an immunocompromised strain of mice suitable for xenograft studies retained their insulin secreting properties, and possessed a mitogenic response to a pharmacologically relevant infusion of Exendin-4. The mitogenic effect of chronic exposure of Exendin-4 in these transplanted mice was observed to arise from stimulation of the calcineurin/nuclear factor of activated T cells (NFAT) signaling pathway, leading to a variety of target genes essential for proliferation (65). Furthermore, this ability to enhance β-cell mass has been recently challenged in a study conducted in

normoglycaemic mice where β-cell mass was decreased following 6 weeks of treatment with Liraglutide (66, 67). Therefore, GLP-1 analogs can have contrasting effects on β-cell proliferation depending on physiological context.

A physiological consequence of T2DM, due to peripheral insulin resistance, is high demand for enhanced insulin protein synthesis in the β-cell. It has been observed that sustained exposure to high insulin synthesis requirement results in ER stress due to protein overload and misfolding (68–72). The unfolded protein response (UPR) is the biochemical program initiated within the cell to counteract the accumulation of unfolded proteins in the ER lumen resulting in destabilization of ER homeostasis (73). The UPR initiates signaling cascades involving the luminal domains of three major ER resident proteins; Inositol requiring enzyme 1 (IRE1), protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6) (71, 74). The pathways so activated via signaling hubs attempt to re-establish ER homeostasis through transcriptional activation of genes involved in protein folding and protein degradation, as well as temporary attenuation of mRNA translation (75–77). This is achieved through downstream signaling events as, phosphorylation of eukaryotic translation initiation factor 2 alpha (eIF2α), resulting in global protein synthesis blockade, and alternative splicing of X-box binding protein 1 (XBP-1), a transcription factor involved in misfolded protein retrotranslocation and degradation (78–80). Failure to alleviate ER stress, and consequent prolonged UPR activation, leads to apoptotic cell death primarily through upregulation of the pro-apoptotic transcriptional factor C/EBP homologous protein (CHOP) (71, 81, 82). Several physiological and environmental insults associated with T2DM have been shown to induce ER stress in β-cells, these include hyperglycemia, dyslipidemia, inflammation and oxidative stress (83).

GLP-1 has been reported to alleviate glucotoxicity, lipotoxicity, excess nitric oxide (NO), Ca^{2+} depletion, oxidative stress, and cytokine-induced ER stress in both primary β-cells and cell lines through several downstream signaling mechanisms (59, 74, 84–86). For example, Yusta, B et al., demonstrated that GLP-1R signaling facilitates the shift from translational repression to translational recovery phase in a PKA-dependent manner (74). The recovery phase is concomitant with enhanced activation of ATF-4, CHOP and over-stimulation of *Gadd34* gene signaling cascade, which leads to eIF2α dephosphorylation. Furthermore, GLP-1 treatment leads to upregulation of spliced XBP-1 (sXBP-1), which is involved, along with ATF6, in enhancing ER function through activation of genes encoding molecular chaperones and ER-associated protein degradation (60–70, 74). In addition, GLP-1 protection from lipotoxic stress has been demonstrated to occur downstream from induction of the ER chaperone Binding immunoglobulin Protein (BiP) and anti-apoptotic protein JunB (86). Animal studies have recapitulated these findings, whereby diabetic mice treated with GLP-1 analogs displayed a significant reduction in biochemical markers of ER stress, increased expression of antioxidant genes and improved metabolic parameters (60, 74).

Most recently, GLP-1 has also been implicated in the regulation of autophagy in β-cells (87, 88). Autophagy, a mechanism that can promote cell survival during nutrient depletion, may also occur under basal and excessive nutrient conditions. This cellular process is characterized by the formation of autophagosomes, which can capture cytosolic components and fuse with lysosomes to promote the recycling and/or degradation of its contents. The process can be separated into four stages, initiation, nucleation, elongation, and fusion/degradation [reviewed in (89)]. Initiation is controlled by the mammalian Target of rapamycin (mTOR)/ AMP-activated protein kinase (AMPK)/ Uncoordinated (Unc)-51-like kinase 1 or 2 (ULK-1/2) axis, a crucial regulatory step leading to the activation of class III phosphatidylinositol 3-kinase complex, formation of the phagophore (a double membrane vesicle that encloses and isolates the cytoplasmic components during autophagy), and ultimately, recruitment of key proteins involved in the nucleation phase (90). Once the phagophore is formed, the elongation phase is undertaken where the phagophore captures the desired cargo for degradation. This is regulated by two ubiquitin-like reactions that act in concert to mediate the localization of key proteins to the developing autophagosome, and expansion of its membrane (91–93). Finally the autophagosome fuses with lysosomes, after which lysosomal enzymes initiate content degradation and nutrient and metabolite recycling (92). Autophagy provides essential components for energy production and biosynthesis during nutrient depletion. However, it also acts in a similar fashion by recycling of damaged organelles, unwanted proteins and foreign matter when adequate nutrients are available (88). In an environment with excessive nutrients, however, autophagy acts to remove unfolded proteins and toxic aggregates, thus facilitating ER homeostasis. GLP-1 can facilitate autophagy under chronic exposure to excess nutrients, whereby it prevents autophagosomal-lysosomal fusion impairment (87, 88). Similarly, Exendin-4 was reported to enhance lysosomal function, consequently leading to improved autophagosome clearance in a rat model of tacrolimus-induced diabetes whereby autophagosome accumulation causes islet injury (94). In the latter study, *in vivo* Exendin-4 treatment decreased tacrolimus-induced hyperglycemia, oxidative stress, and apoptosis. In parallel, it was demonstrated that β-cells from treated animals presented with reduced autophagosome numbers and decreased autophagy related protein expression. Thus, GLP-1R signaling could be interpreted as inhibiting autophagy, however, it most likely depicts its positive effects on autophagosomal-lysosomal fusion and, therefore, as a positive mediator of autophagic flux. It should be noted, however, that GLP-1 induced changes can vary depending on the underling mechanism of stress. For example, while usually promoting autophagy, treatment with GLP-1 analogs in a high fructose fed rat model resulted in apparent inhibition of β-cell autophagy, and increase in β-cell mass and function (95). The underlying mechanisms and downstream molecular mediators through which GLP-1 influences autophagy remain to be better characterized.

Recently, several studies have significantly improved our understanding of the regulation of β-cell energy metabolism by chronic GLP-1R activation. Notably, acute vs. chronic effects of receptor activation and downstream cAMP signaling leads to two distinct waves of gene expression regulation in primary

islet cells. The initial wave of gene expression occurs as rapidly as 2 h after cAMP elevation upon acute receptor activation, and is mediated by CREB. Sixteen hours from the initial stimulation, a second wave of gene expression regulation takes place, and is orchestrated by *Hypoxia-inducible factor 1* (HIF-1), a transcriptional factor that targets genes involved with glucose uptake and glycolysis (21). Van de Velde et al. reported that chronic GLP-1R activation led to metabolic reprogramming marked by increased ATP production and upregulation of glycolytic enzymes, occurring as a result of late activation of subunit alpha of HIF-1 (HIF-1α) downstream of mTOR. This finding is consistent with a recent study demonstrating that depletion of HIF-1α or inhibition of mTOR impaired the effects of GLP-1R signaling on glycolysis (18). Whilst it is known that the Phosphoinositide 3-kinase (PI3K) /AKT (Protein kinase B) /mTOR axis is intimately linked to metabolic functions such as protein synthesis, glucose uptake, ATP production, nutrient transport, autophagy and cellular growth (96, 97), recent publications have identified novel effects of chronic GLP-1R stimulation which impact these pathways. For instance, GLP-1R agonists have been reported to promote secretion of insulin like growth factor-2 (IGF-2), and induced expression of its receptor (IGF1-R), which once stimulated activate downstream cascades including the PI3K/AKT as well as the cellular growth and proliferative mitogen-activated protein kinase (MAPK) pathway (19, 20, 98). The biosynthesis, secretion and subsequent activation of the IGF-2/IGF-1R autocrine loop is significantly enhanced by the presence of glutamine, and has been reported to protect β-cells against apoptosis, and increases β-cell glucose competence (20, 98, 99). However, whilst this autocrine loop stimulates PI3K/AKT activity and contribute to some of the pro-survival abilities of GLP-1 in β-cells, it does not seem to mediate the enhanced metabolic phenotype induced by chronic exposure to GLP-1R agonism. Rowlands et al. demonstrated that neither functional inactivation of IGF-2 nor silencing of its receptor by siRNA could mitigate the observed metabolic adaptations enacted by prolonged exposure to Exendin-4 (19). Thus, as the mitochondria and ER form structural and functional networks, the ability of GLP-1 to enhance metabolism may reduce ER stress by enhancing mitochondrial derived ATP and Ca^{2+} for utilization in the maintenance of ER homeostasis (46, 100, 101). Through elevation of cytosolic and intra-mitochondrial Ca^{2+}, and mobilization of intracellular Ca^{2+}, GLP-1 may mitigate the required Ca^{2+} transfer from ER to mitochondria, thereby sustaining mechanisms which facilitate protein folding (102–104). Therefore, we hypothesize that metabolic reprogramming in β-cells underlies the protective effects of GLP-1 under various stress conditions. In this scenario, enhanced metabolism can provide additional energy required to facilitate stress response and pro-survival mechanisms utilized by β-cells during challenging physiological conditions.

Although primarily studied in the pancreatic β-cell, the beneficial effects of GLP-1 and its analogs have recently been shown to be advantageous to a variety of tissues in several disease pathologies, such as the heart, liver, lung, muscle, and brain, as detailed below in the following sections. It remains to be clarified, however, as to whether the metabolic and pro-survival responses arising from chronic GLP-1R mediated signaling cascades are relevant to extra-pancreatic tissues as well.

GLP-1 ACTION IN OTHER TISSUES

Skeletal Muscle

Beneficial actions of GLP-1 differ between, skeletal, smooth and cardiac muscle, and again between the two subsets of smooth muscle, single and multi-unit cells (gastrointestinal/urogenital and vasculature cells, respectively) (105). Due to its ability to act on numerous pathways that can regulate glycaemia, weight, lipid metabolism, and blood pressure **(Figure 2)** (1, 29), as further outlined below, GLP-1R agonists, have been implicated and implemented as a potential therapy to address the increasingly prevalent pathologies associated with metabolic syndrome.

Studies assessing GLP-1's extra-pancreatic effects, such as its insulin-like actions, revealed that exposure of skeletal muscle to GLP-1R agonists enhanced glycogen synthesis, glycogen synthase α (GSα) activity, glucose metabolism and inhibited glycogen phosphorylase α activity in diabetic and non-diabetic rodent models, and human tissue (106–109). Interestingly, compelling evidence suggests that such muscle effects are independent of cAMP signaling. This was observed in rat and human muscle cells, but also in studies conducted in hepatocytes and adipocytes, potentially utilizing inositol phosphoglycans (IPGs) as the intracellular second messenger (108, 110, 111). While the amino acid sequence, same as that of the pancreatic GLP-1R, has been identified in multiple tissues (112), these studies identified that GLP-1 acted through a unique receptor distinct from that of the β-cell, which allowed this deviation from the canonical signaling of GLP-1. Whether this deviation from the non-canonical effects of the GLP-1R in muscle tissue are resultant from alternative splicing, the widespread hetero dimerization of B-family GPCRs, variations in ligand-receptor interactions or GLP-1 degradation products still requires further investigation (113–115).

To date, understanding of GLP-1 effects in skeletal muscle has mostly stemmed from the laboratory of Villanueva-Penacarillo, who have revealed that GLP-1R agonists can induce PI3K/PKB (Akt), P44/P42 MAPK, p70S6K, and Protein kinase C (PKC) signaling pathways in skeletal muscle cells (116–119). Corroborating this findings, similar results were obtained in L6 myotubes and 3T3-adipocytes, that Exendin-4 promoted a PI3K dependent increase in insulin-stimulated glucose uptake (120). Other lines of evidence suggest that GLP-1R activation promotes skeletal muscle glucose transport independent of insulin through the AMPK signaling pathway and downstream activation of TBC1D1, a paralog of the phosphorylated Akt substrate AS160, thereby leading to translocation of GLUT4 to the plasma membrane (121–124). Akt is the canonical mediator of insulin-induced GLUT4 translocation. Although, it should be noted that the difference in signaling pathways from these latest studies may result from the extended duration of exposure to the GLP-1R agonist (122, 123). Interestingly, two of the above mentioned papers reported a rise in cAMP measured in muscle cells, as well as an increase in PKA contributing to a favorable metabolic phenotype in the studied muscle cells (121, 123). One

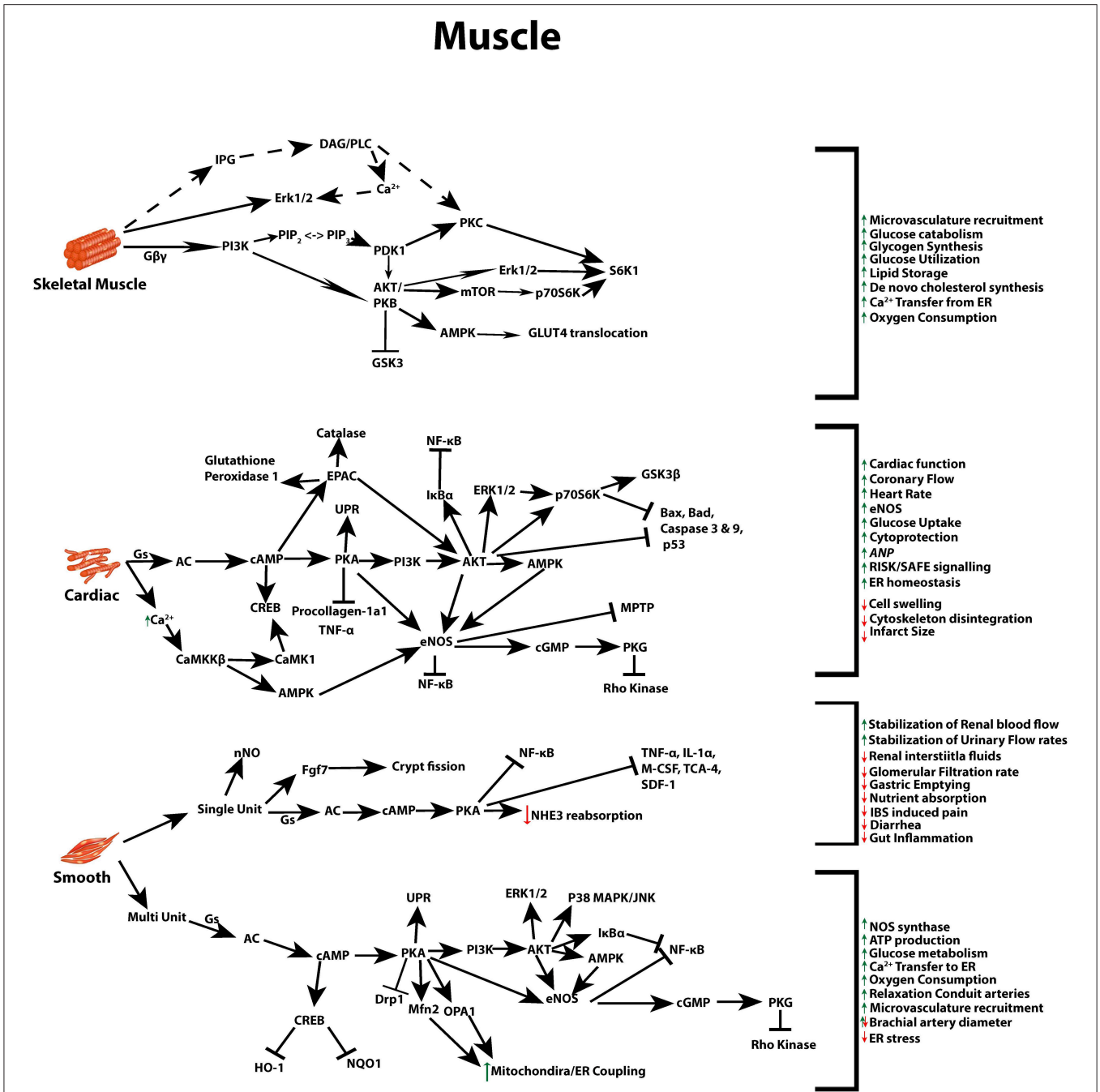

FIGURE 2 | GLP-1 signaling across different muscle types. Multiple signal transduction pathways and their net impact arising from GLP-1R stimulation in skeletal, cardiac, and both multi and single unit smooth muscle.

possible explanation for these findings may be that, as in neurons and sperm, increases in cAMP could be a result of enhanced Ca^{2+} or even calcium/calmodulin-dependent protein kinase II activity which in turn can activate AC (125–128). While the ability for GLP-1 to impact skeletal muscle in regards to glucose catabolism and glycogen synthesis has been analyzed in depth, further mechanistic studies are required to fully elucidate the role for incretin hormones in regard to this tissue type.

Smooth Muscle and Vascular Tissue

Recent studies have described receptor dependent and independent effects of GLP-1 in smooth muscle, whereby exposure to physiological concentrations of acutely infused GLP-1 can relax conduit arteries in healthy humans, and recruit skeletal and cardiac muscle microvasculature. Dilatation of microvessels can facilitate insulin and nutrient delivery, tissue oxygenation, and glucose utilization (129, 130). This dilatory

effect is believed to occur as a result of GLP-1 binding to the abundantly expressed, endothelial cell GLP-1R, triggering a downstream signaling cascade resulting in microvasculature recruitment via a NO-dependent mechanism (129, 131, 132). Additionally, the effect of the GLP-1 analog Liraglutide on endothelial cells has been evaluated in cultured human umbilical vein endothelial cells (HUVECS) to evaluate its impact on ER stress and apoptosis induced following overnight exposure to high glucose (133). The authors found that such treatment reduced apoptosis and ER stress through a mechanism which likely involves stimulation of the nuclear-encoded mitochondrial protein optic atrophy protein 1 (OPA1). It appears that the ability for GLP-1 to modulate mitochondrial metabolism is not limited to the β-cells. Indeed Morales et al. (134) reported that GLP-1 treatment stimulates mitochondrial activity in A7r6 vascular smooth muscle cells through recruitment of the ER to the mitochondria via the tethering protein Mitofusin-2 (Mfn-2). Enhanced Mfn-2 promotes ER-mitochondria co-localization and increases Ca^{2+} transfer from ER to the mitochondria, thus facilitating high demand for oxygen consumption and ATP production.

Studies conducted in rodent models have shown that stimulation of the GLP-1R led to changes in blood pressure (BP), depending on experimental model (29, 135, 136). In humans, one study reported an increase in BP over 2 h from healthy subjects following a single administration of GLP-1 (136), while another reported that chronic administration of GLP-1 analogs to patients with metabolic syndrome led to a reduction in BP [reviewed in (29, 137, 138)]. Such conflicting results may result from complex actions of GLP-1 on vascular smooth muscle and cardiac tissues in combination with its effects in the autonomic nervous system. Altogether GLP-1's multi-tissue actions can mediate alterations in BP, vasodilation and constriction, body weight, and heart rate (120). Nevertheless, the short- and long-term effects of GLP-1 on vascular smooth muscle are not completely understood and thus require further examination to ensure GLP-1 therapies can be utilized to their utmost potential.

Another mechanism by which GLP-1 therapies have been utilized to attenuate or partially attenuate metabolic syndrome is through their impact on diet and satiety (29, 130, 133, 139, 140). Notably, GLP-1's action on gastric emptying has been indicated to be enacted by reduced contraction in human intestinal muscles, and it occurs as a direct result of activation GLP-1R in the gastrointestinal (GI) tract (140), rather than for its ability to mitigate food motivated behavior through receptor activation in the hypothalamus or the hindbrain (141). However, the effects on gastric emptying appear to be short acting, since clinical studies and animal models of chronic administration of GLP-1R activators altogether suggest a negligible effect on long-term gastric emptying. Instead, evidence suggests that reduced weight gain occurs through direct actions on the pancreas, as well as through reduction of appetite mediated by central nervous system responses (141–143). Reducing the rate of gastric emptying, however, does not just impact satiety, but also delays the rate of entry of nutrients into the small intestine and their subsequent absorption, which therefore influence postprandial glucose metabolism, hormonal responses, and ultimately enhances GLP-1's anti-diabetogenic effects (144, 145). Slowing of small bowel motility was reported to occur in a GLP-1R and nitric oxide (NO) dependent manner, independently of both somatostatin and insulin, in fasting but not fed rats (146). Such effects have been reported in healthy (147), obese (148), diabetic (149), and critically ill human subjects (150). While gastric relaxation and postprandial gastric accommodation were reported to be mediated by vagal cholinergic pathways (151–153). Work by Amato et al. (154) validated these findings by demonstrating that acute administration of GLP-1 activated the GLP-1R in human colon cells and resulted in an inhibitory effect on large intestine motility through release of neural NO.

Kidneys

A broad array of renoprotective properties have been reported from GLP-1 therapies. Positive effects in the renal tissue were observed both in diabetic and non-diabetic models of chronic kidney disease (CKD), as well as acute kidney injury (AKI) (155–160). Although not often considered as part of the metabolic syndrome, accumulating evidence has begun unearthing a link between the increasing morbidity and mortality rate in patients with kidney disease and metabolic syndrome (156, 159, 161, 162). This entwinement of kidney disease and metabolic syndrome complicates investigations with GLP-1 therapies due to the indirect benefits GLP-1R agonist therapies have on other tissues including but not limited to alterations in BP, glucose homeostasis, weight loss and insulin levels [reviewed in (155, 156, 161, 163)]. Adding to this scenario is the lack of agreement regarding the exact locality of GLP-1R expression in the kidney (1, 164), although it is generally accepted that in humans and rodents the GLP-1R is expressed in the renal vasculature and afferent arterioles, with some studies reporting receptor expression in the proximal tube and glomerular capillary, but not in the distal tubules (155, 165–168). It is evident from both clinical and animal studies that GLP-1 based therapies are beneficial to kidney function through increases in renal blood flow (RBF), urinary flow rate, prevention of rises in plasma creatinine, reduced tubular necrosis, an increase in renal interstitial fluids and glomerular filtration rate (GFR), as well as cytoprotective and anti-inflammatory actions (160, 161, 164, 166, 169, 170).

GLP-1R agonists are believed to cause these effects in the kidney through both direct kidney based GLP-1R activation, and indirect receptor actions, potentially through interactions with the nervous system (170), the renin angiotensin system (RAS) (155, 171–173), and regulation of atrial natriuretic peptide (ANP), a blood pressure and electrolyte regulator (173). Regardless of this lack of consensus in terms of indirect kidney responses to GLP-1 therapies, the direct actions of GLP-1R activation in the renal tissue are consistent. Acute exposure increases the diuretic and natriuretic excretion rate, which is in part dependent on inhibition of $NaHCO_3$ reabsorption via a cAMP/PKA modulation of NHE3 (renal cortical Na^+/H^+ exchanger isotope 3) (166, 169). Furthering this, GLP-1's renal hemodynamic actions have been observed to alter GFR, potentially to regulate the filtered electrolyte load and volume (159, 174). In this sense, Exenatide acutely increased GFR

and suppressed proximal tubular reabsorption in Wistar rats, resulting in approximate doubled early distal flow rate (175). Altogether these finding imply that Exenatide works as a diuretic at the kidney level. Similar results have been recently reported in humans (176).

Activation of the GLP-1R/cAMP/PKA pathway is also crucial in renal protection, with studies in a range of rodent models reporting a reduction in renal inflammation, renal fibrosis, and decrease in renal oxidative stress arising from the toxic milieu induced in metabolic syndrome (158, 161, 166, 177, 178). These pro-survival abilities are believed to arise from enhanced GLP-1 signaling leading to a reduced expression of the pro apoptotic markers caspase-3, and Bax/Bcl-2 (158), as well as reducing oxidative stress through increased expression of the oxidative defense gene heme oxygenease-1 (HO-1) (160, 178), and inhibition of NAD(P)H oxidase in a cAMP/PKA dependent manner (166). Activation of the GLP-1R signaling pathway has also been reported to reduce macrophage infiltration, potentially alleviating the associated increase in ROS and inflammation, as well as attenuating the progression of renal fibrosis through downregulation of ERK1/2 and its upstream activator transforming growth factor-beta 1 (TGF-β1) (160, 177). Despite these results, further studies are still required to fully elucidate the molecular mechanisms that mediate the reported attenuation in apoptotic and inflammatory pathways, particularly as accumulating clinical evidence highlights the potential of GLP-1R agonist therapies in DKD, ultimately urging for deeper understanding of cellular actions of these analogs in the renal tissue [reviewed in (156, 157, 159, 162, 179)].

Adipose Tissue

Although GLP-1 based therapies primarily aid weight loss through satiety, their usefulness is further extended by multiple studies implicating GLP-1R agonists as regulators of adipogenesis. Studies have indicated that GLP-1 based therapies can potentially influence whole body energy metabolism through their regulation of adipocyte development, acceleration of plasma clearance of glucose and triacylglycerol derived fatty acids, improvement of insulin signaling and stimulation of brown adipose tissue (BAT) thermogenesis (31, 142, 180–184). The GLP-1R in adipocytes was reported to activate the AC/cAMP signaling pathway, regulating apoptosis and pre-adipocyte proliferation through various cell signaling cascades including ERK, PKC and AKT, as well-altering the expression of peroxisome proliferator-activated receptor gamma (PPARγ) and its target genes (142, 185). GLP-1 may also act through a brain-adipocyte axis to modulate lipid metabolism in BAT, as well as white adipose tissue (WAT). In various rodent models, administration of GLP-1R agonists induced BAT thermogenesis through increased uncoupling protein 1 (UCP1), mitochondrial respiratory chain element *Cox4i1 (*Cytochrome C Oxidase Subunit 4I1*)* and PGC1α, independent of nutrient intake, as well as altering the expression of transcription factors involved in *de novo* lipogenesis (123, 181, 186). Interestingly, GLP-1 has also been shown to activate Adipose-resident invariant natural killer T (iNKT) cells, triggering fibroblast growth factor 21 (FGF21), a major player in iNKT cell induced weight loss (187).

While still in its infancy, and convoluted by the various interconnected pathways, studies investigating the effects of GLP-1 therapies in the adipose tissue of patients with obesity show promise, with trials replicating *in vitro* studies, and indicating a potential long term benefit of GLP-1R agonists therapies also in this important tissue. Deeper studies into the underlying mechanisms are warranted in order to specifically identify direct actions of GLP-1 agonists in BAT and WAT physiology and lipid metabolism.

Heart

Given that both T2D and obesity represent important risk factors for cardiovascular disease (CVD), there is emerging interest to establish the potential cardiovascular benefits of GLP-1R stimulation. Even though the positive effects of GLP-1 analog therapies on the metabolic conditions described above could theoretically improve CVD outcomes, mounting evidence points that GLP-1 can also influence the cardiac tissue through direct receptor mediated responses. Indeed, it is recognized that the classical response initiated by GLP-1R activation leads to facilitation of cardiac function through enhanced glucose uptake, improved coronary flow, and in mice, secretion of atrial natriuretic peptide (ANP), a blood pressure and electrolyte regulator (163, 173, 188). However, studies to define the mechanism through which GLP-1 directly influences cardiac tissue are complicated by its broad actions in other tissues, such as blood vessels. For instance, a study conducted by Mells et al. (189) indicated that liraglutide treatment was able to reverse BP increases and cardiac hypotrophy resulting from a high fat diet (HFD) induced obese mouse model. The study, however, did not dwell further into the underlying mechanisms, making it particularly difficult to distinguish between the direct effects of the treatment in the cardiac muscle from those emanating from other tissues. Recent data published by the Drucker Laboratory and colleagues have indicated that some of the contrasting results of GLP-1 on the cardiovascular system in regards to both increasing and decreasing heart rates, and BP, are partially mediated by neurological signaling (120). Remarkably, in studies where the GLP-1R was conditionally disrupted only in mice cardiomyocytes ($GLP\text{-}1R^{CM-/-}$), pre-treatment with liraglutide could still promote cardioprotection, increased survival and reduced infarct size following ischemia-reperfusion injury, suggesting these outcomes are not mediated directly by cardiomyocyte GLP-1R activity (190). Glucagon-like peptide (GLP)-1 (9–36) amide-mediated cytoprotection in ischemic-perfused mice was blocked by the GLP-1R antagonist exendin-9–39 but did not require the known GLP-1 receptor (190–193). Thus, the direct and indirect mechanisms which underpin the beneficial effects of GLP-1R agonism on cardiac injury remain to be clarified.

In an effort to address these gaps in knowledge, multiple groups have endeavored to define the role of GLP-1, its analogs, and related peptides (11, 194), in protecting cardiomyocytes and endothelial cells from injury. Indeed, GLP-1R activation leads to the re-establishment of ER homoeostasis, cytoprotection, and

restoration of signaling pathways disrupted by diverse stress stimuli (139, 191, 192, 195). For example, liraglutide treatment corrected the decreases in eNOS, the endothelial nitric oxide synthase, responsible for most of the vascular nitric oxide production in a HFD model of cardiac dysfunction in mice (195). This is of particular importance as NO is crucial in a pathway that regulates the synthesis of the ubiquitous intracellular second-messenger cyclic guanosine 3′,5′-monophosphate (cGMP). It has been reported that cGMP can activate two types of effector molecules in cardiovascular system, cGMP-dependent protein kinases (PKGs) and phosphodiesterases (PDE), which can stimulate cellular proliferation, mediate vaso-relaxation, and inhibit hypertrophy (196). Importantly, the effects in eNOS were accompanied by significant decreases in cardiac tissue TNF expression and NFκB activation (195). These effects were confirmed to be direct actions of the GLP-1R agonist in heart and vascular tissues since liraglutide also prevented palmitate-induced lipotoxicity in isolated mouse cardiomyocytes and primary human coronary smooth muscle cells *in vitro*. Together these data indicate that GLP-1R activation can activate multiple complementary protective and pro-survival mechanisms in cardiac cells, and endothelial cells. These findings are further supported by larger animal trials in which GLP-1 induced reduction in infarct size after ischemia-reperfusion (IR) injury (197), improved left ventricular function, and altered heart rate and BP when infused into dogs with pacing-induced cardiomyopathy (198). Furthermore, GLP-1 is hypothesized to activate ischemic conditioning (IC) through reperfusion injury survival kinase (RISK) and survivor-activating factor enhancement (SAFE) pathways (199, 200). Activation of this conditioning pathway post GLP-1R stimulation has been shown to reduce infarct size, improve cardiac function and enhance AKT activation and Bcl-2, an important anti-apoptotic protein, expression after IR injury in pigs (201). IC is interconnected with the mitochondrial K-ATP channel (mK-ATP) (202, 203) as well as the ATP derived metabolite adenosine, which activates the adenosine receptor and its signaling pathway leading to ischemic preconditioning (193, 204, 205). Although still unclear, the role of GLP-1R signaling cascades in the activation of conditioning pathways may include hijacking these subcellular pathways. Furthering this, the GLP-1 mediated relaxation of *ex vivo* rat aorta described by Green et al. (194), was lost upon K-ATP channel blockage, indicating a link between GLP-1R activation induced IC.

Initial human trials mimicked cellular and animal model studies, with GLP-1 therapies improving left ventricular (LV) function in patients with acute myocardial infraction (AMI) and serve systolic dysfunction (206). The promising results of this pilot study were followed in 2006 by an additional study demonstrating that chronic GLP-1 infusion over 5 weeks can improve LV function and quality of life in diabetic and non-diabetic participants (207). As these improvements were seen in both diabetic and non-diabetic groups, glycemic control in the GLP-1 treated group was deemed not to be a contributing factor to the beneficial effects. Since these early studies, treatment with GLP-1 analogs has been noted to improve hemodynamic recovery in patients undergoing coronary artery bypass grafting (208), protect against ischemic LV dysfunction (209), prevent hyperglycemia during cardiac surgery (210) and reduce reperfusion injury (211–213). Finally, data from recent large scale cardiovascular outcomes in T2DM trials revealed a significant reduction in cardiovascular death rates in GLP-1 analog treated patients (214, 215). Research into GLP-1 therapies on cardiac tissue continues to represent an expanding field, with the potential for a broad range of therapeutic applications beyond cardiovascular outcomes to be realized through an understanding of the underlying mechanism of action.

Liver

Of the body's organs, levels of GLP-1 are recognized to be highest in the liver owing to transport of the incretin through the hepatic portal vein. The therapeutic effect of GLP-1 and its analogs on restoring hepatic function impaired by a variety of insults is supported by *in vivo* and *in vitro* studies (27, 216). Changes induced from GLP-1 or its analogs, in the liver, regulate a variety of processes including, hepatic gluconeogenesis, glycogen synthesis, and glycolysis **(Figure 3)** (1, 216, 217). In rodent models, GLP-1R agonist based therapies have been reported to increase both glycogen and glycogen synthetase α, through PI3K, PKC, PP-1 (type 1 protein phosphatase), pathways in isolated hepatocytes (218), as well as acting in an insulin-like manner to inhibit glucagon-induced glycogenolysis in perivenous hepatocytes (219). While the presence of the GLP-1R is still controversial in hepatocytes, GLP-1R expression at the protein level has indeed been reported in transformed human hepatocyte cell lines, HuH7 and Hep-G2, as well as primary human hepatocytes (220). However, regardless of the presence of the receptor in hepatocytes, direct receptor-ligand mediated actions in the liver remain controversial with some research groups proposing that observed benefits are a result of receptor independent events (221–225). Mechanisms may include GLP-1 degradation products GLP-1_{9-36}, GLP-1_{28-36} or GLP-1_{32-36} which may be transported through the plasma membrane without the involvement of a receptor, and activate AC and Wnt signaling [reviewed in (226)].

To date, studies in animals and humans have provided evidence for the potential of Liraglutide to improve hyperlipidemia, liver fibrosis and inflammation, non-alcoholic fatty liver disease (NAFLD), as well as reduce liver fat content in T2DM patients (227–230). Acute exposure of Sprague Dawley (SD) rats to GLP-1R activators controlled hepatic glucose production (HGP) through a gut-brain-liver neuronal axis, discussed later, involving GLP-1R stimulated duodenal mucosal PKC-δ activation (231). In this context, Exendin-4 was found to inhibit key gluconeogenic enzymes and enhance hepatic insulin signaling. Exendin-4 was also reported to improve hepatic steatosis and insulin sensitivity in ob/ob mice, which was paralleled by reduction in oxidative stress and genes associated with fatty acid synthesis (232). Female APOE*3-Leiden.CETP mice, a model with human-like lipoprotein metabolism, were fed a cholesterol-containing diet and subsequently treated for 4 weeks with exendin-4. Utilizing a mouse model with human-like lipoprotein metabolism and western-type diet for 5

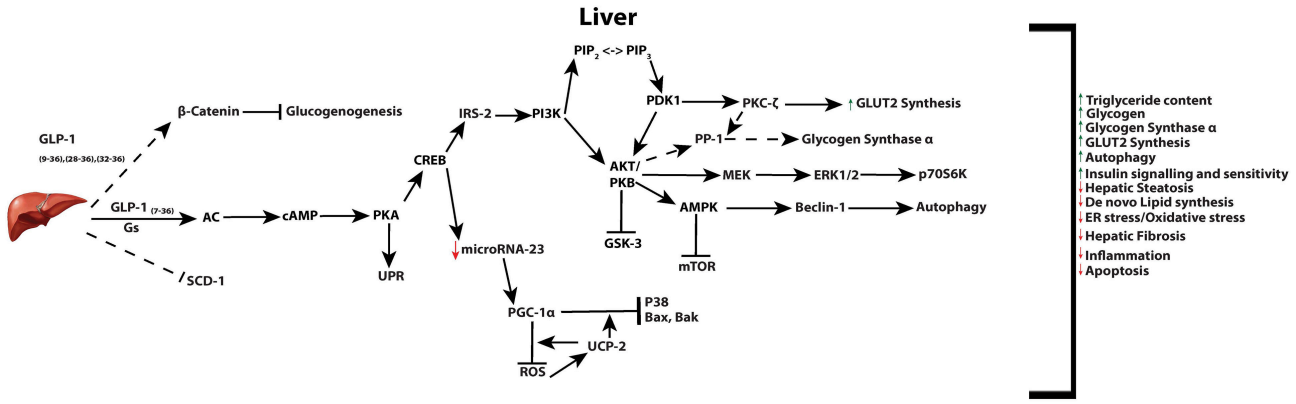

FIGURE 3 | Active GLP-1 and its truncated products signaling in the liver. Active GLP-1 and its cAMP dependent pathways (solid arrows), as well as hypothesized (dashed arrows) truncated product induced signaling and their respective net effects.

weeks to induce atherosclerosis, Exendin-4 treatment reduced inflammation within the liver and vessel well (233). Use of Exendin-4 in this mouse model limited the progression of hepatic inflammation and atherosclerosis through a reduction in macrophage influx and adhesion to the liver and vessel wall. These findings are further supported by a study revealing that GLP-1 analogs impact the production of triglyceride-rich lipoproteins in normoglycaemic men (234).

The hepatic actions of GLP-1 may be mediated through signal transduction of the AMPK/mTOR pathway. This was reported in a study showing that improvement of hepatocyte steatosis by liraglutide involves autophagy and its controlling AMPK/mTOR pathways (235). By inducing autophagy, GLP-1 therapies can relief the burden in the ER, reduce ER-stress, and subsequent hepatocyte apoptosis (236). Understanding the impact of GLP-1 treatments on the liver is crucial, as perturbations to both cellular lipid and very-low-density lipoprotein (VLDL) metabolism are associated with development of hepatic insulin resistance, obesity and diabetes. Recently, chronic stimulation of the GLP-1R led to increases in the mitochondrial uncoupling protein 2 (UCP2), an anti-mitochondrial oxidative stress gene, and the master mitochondrial biogenesis regulator and protective gene, peroxisome proliferator activated receptor-gamma coactivator 1α (PGC-1α) (237). Such gene expression changes were hypothesized to be mediated through downregulation of the microRNA-23 and result in improved hepatocyte survival through reduction in mitochondrial ROS production, inhibition of P38 activity, and decrease in expression of apoptotic genes Bak and Bax (237, 238). Combined with previous knowledge that PGC-1α and UPC2, play critical roles in mitochondrial metabolism (239), these data provide additional support for the hypothesis that the improved metabolism resultant from GLP-1R stimulation underlies the pro-survival abilities of GLP-1 signaling pathways.

Brain

GLP-1 and its receptor agonists are able to influence a variety of brain functions, including but not limited to: satiety, thermogenesis, blood pressure, neurogenesis, neurodegeneration, retinal repair, and altering energy homeostasis **(Figure 4)** (26, 30, 240–245). The GLP-1R is expressed in cells of the cerebral cortex, hypothalamus, hippocampus, thalamus, substantia nigra, circumventricular organ (CVO), cerebellum, and brainstem nucleus. This pattern of gene expression in the nervous system is evident in rodent, non-human primates, as well as humans (26, 241, 242, 244, 246–248). Studies with mice have identified the source of GLP-1 to derive from preproglucagon neurons of the nucleus of the solitary tract within the brainstem (249). These neurons project to the thalamus, hypothalamus and cortical regions, and induce the release of GLP-1 by various stimuli in a mechanism similar to L-cells of the small intestine (30, 31, 240, 243). Gut-derived GLP-1 can cross the blood brain barrier (BBB) and bind receptors in the circumventricular organs of the brainstem, however its short half-life is believed to limit its function within the brain. Instead, it most likely influence the brain indirectly, through vagal nerve fibers in the enteric area, whereby it transmits metabolic information to the nucleus of the solitary tract (NTS)—neurons responsible to control brain regions known to mediate feeding behavior (250). Recent research has revealed that GLP-1 analogs, due to their extended half-lives, can reach the BBB and have distinct effects to endogenous GLP-1 in the brain (26, 250). These effects include the well-investigated anorexigenic effects, outlined below, as well as a range of neuroprotective abilities that have led to the use of GLP-1R agonists as therapies in human trials for a range of neurodegenerative diseases as discussed in detail further below.

Studies in rats using intracerebroventicular (icv) administration of GLP-1 or its analog Exendin-4, alone or in combination with the receptor antagonist Exendin (9–39), have shown that activation of the GLP-1R inhibits food intake and weight gain; such effects are attributed to changes in brain controlled hormone secretion (251–254). Similar findings have been recapitulated in studies of obesity in humans, and have highlighted GLP-1 based therapies as potential anti-obesity treatments. The role for GLP-1 signaling in satiety is understood to be a consequence of GLP-1R signaling attenuating the release of the orexigenic neuropeptides Neuropeptide Y (NPY)

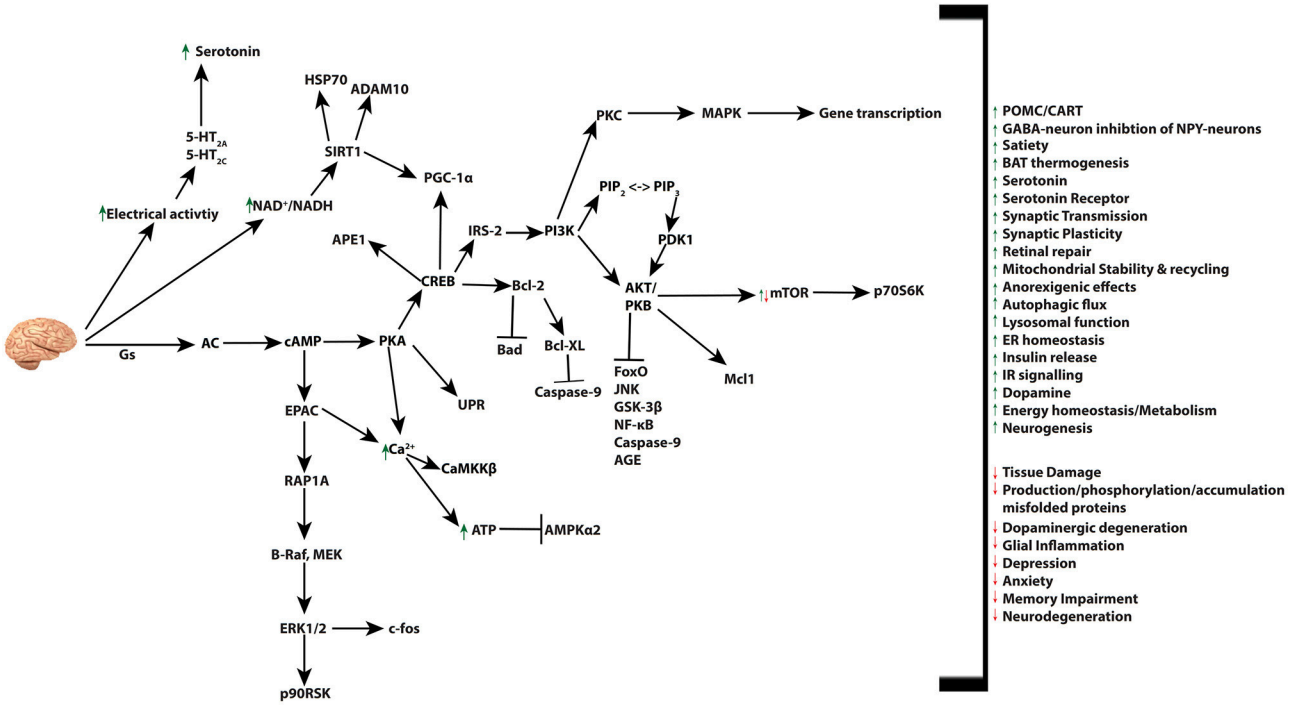

FIGURE 4 | Pleiotropic effects of GLP-1 signaling in the brain. GLP-1 and its analogs activate diverse signaling pathways in the brain, leading to a plethora of neuroprotective outcomes.

and agouti-related peptide (AgRP), as well as promoting the anorexigenic neuropeptides pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) (250, 254–256). These neuropeptides are produced by arcuate nucleus of the hypothalamus (ARC), a critical regulator of energy balance, feeding behavior, and body weight (257, 258). GLP-1 is believed to alter food intake through this pathway, with an acute receptor induced modulation of AMPK activity in the hypothalamus. Secher et al. (259), supports these findings, but also discovered that while the GLP-1 analog Liraglutide directly stimulates POMC/CART neurons, it indirectly inhibits NPY/AgRP neurotransmission via GABA-dependent signaling. This liraglutide induced weight loss, altered food intake and conditioned taste aversion occurs through CNS receptors rather than the vagus nerve, area postrema, paraventricular nucleus, or visceral nerves (259, 260). Novel data has recently surfaced proposing that activation of astrocyte GLP-1Rs may play a role in energy balance in the CNS and GLP-1s anorectic effect, although the mechanics underlying this new finding still require deeper investigation (261). Furthermore, native GLP-1 infusions in the CNS, have been shown to modulate thermogenesis of BAT, via enhanced sympathetic nervous system (SNS) activity (183). Both chronic and acute CNS infused GLP-1 promoted BAT activation and subsequently glucose and triglyceride uptake, via activation of AMPK in the hypothalamic ventromedial nucleus in rodents (181, 182). GLP-1 is also able to upregulate alternatively activated (M2) macrophage-related molecules in human monocyte-derived macrophages (HMDM). This M2 macrophage activation enhanced production of anti-inflammatory factors was also found noted to enhance adiponectin secretion from adipocytes and derived from GLP-1 induced activation of the activator of transcription 3 (STAT3) which can further contribute to its protective abilities against metabolic syndrome (262). More recently, GLP-1 and its analogs have been shown to act in the dorsal raphe, whereby GLP-1R activation alters serotonin turnover and the 5-hydroxytryptamine 2A (5-HT_{2A}) and 5-HT_{2c} serotonin receptors in rats (263). Dorsal Raphe GLP-1R stimulation induces hypophagia and increases the electrical activity of the serotonin neurons in this region, indicating that serotonin may be a new neural substrate for GLP-1 activity and aid GLP-1s ability to reduce appetite, and body weight through bioenergetics metabolism (263). Whilst the ability of GLP-1 and its analogs to stimulate serotonin receptors in humans has yet to be tested, various studies have employed neuroimaging techniques, such as functional magnetic resonance imaging (fMRI) to assess GLP-1 actions in human brain. These studies recapitulated animal studies, where infusion of GLP-1 analogs was found to; attenuate neuronal activity in reward processing areas, reduce appetite and hedonic feeding in healthy volunteers (264), as well as obese and T2DM patients

(265, 266). Whilst weight loss as a result of GLP-1 therapies in humans is believed to be primarily resultant from their inhibitory effect on food intake; distinct neuronal responses should be taken into account when investigating the effects of GLP-1 in neurodegenerative diseases. Chronic inflammation of the brain is a known pathophysiological hallmark of various neurodegenerative diseases, including Alzheimer's disease (AD), multiple sclerosis (MS), and Parkinson's disease (PD), all demonstrated in animal models to benefit from GLP-1 mimetics therapy (14, 26, 30).

Mental illnesses and neurodegenerative diseases not only negatively impact on a patient's quality of life through their impairment of motor functions, they can enhance dementia and depression, which are often refractory to treatment (267). This resistance to treatment may be influenced by accumulating evidence that implicates a link between neural inflammation and the pathology of depression (268, 269). In addition, analysis of patients with psychiatric illnesses has revealed an alteration to crucial intracellular signaling pathways including the AKT/GSK-3 (glycogen synthase kinase) pathway (269, 270). This data along with observation that GLP-1R signaling enhanced levels of serotonin, dopamine (DA), and their receptors (263, 271), has potentiated the use of GLP-1R agonists as a management strategy for mental illness and neurodegenerative diseases. While initial *in vitro* studies have begun to reveal the mechanistic effects of GLP-1 in regards to neurodegenerative diseases, as outlined further below, both animal and human trials have already taken advantage of GLP-1's beneficial actions. Several animal studies have been undertaken with two studies showing that chronic treatment in rats is associated with reversal of depression-like behavior and acute treatment induced anxiety-like behavior (270, 272), while an alternate study indicated no GLP-1 induced changes in either behavior (273). Such contrasting results raise caution in the design and implementation of human trials which evaluate the therapeutic effects of GLP-1 and its analogies in the treatment of neurodegenerative diseases.

Although several recent studies have begun to elucidate the impact of GLP-1 analog therapies on the progress of neurodegenerative disorders, no human clinical trials have directly measured the impact of GLP-1 analog therapies on mental health disorders. Despite this, several trials using GLP-1 analogs for T2DM have a battery of neuropsychological tests as secondary outcome measures that may provide insights into GLP-1's impact on mental illness (274). Whilst many studies are still underway, only one recent clinical study analyzing the effect of the liraglutide analog in regards to Alzheimer's disease has been reported (NCT01469351) (275), and two clinical trials and one population based nested case-control study assessing Parkinson's Disease and GLP-1R activity (NCT01971242, NCT01174810) (28, 267, 276–278). Findings from the AD study in Denmark indicated that GLP-1 analog treatment caused a slight, but non-significant increase in cerebral glucose metabolism (CMR_{glc}) after 6 months of treatment (275). As a decline of CMR_{glc} correlates with cognitive impairment, synaptic dysfunction and evolution of the disease, GLP-1's slight reduction as noted in this study offers a potential mechanism of benefit. However, a large gap in regards to AD and GLP-1 based therapies remains, since the small sample size of the study precluded the ability for the study to determine if GLP-1 administration reduces amyloid β (Aβ) load or alter cognitive scores (275). Nevertheless, in PD, an initial study into GLP-1 analog therapy, published in 2013, assigned 45 patients with moderate PD to receive subcutaneous Exenatide injections for 12 months alongside patient which did not receive any injection. Despite lacking a placebo-control in this study, the blinded ratings were indicative of clinical improvement in both motor and cognitive measures compared to control (277, 278). This study has since been expanded with a Swedish group assessing the effects of both GLP-1R agonists and DPP-4 inhibitors, and a UK based study opting for once weekly Exenatide treatments. The Swedish study, through use of a population-based nested case-control study, found a significantly decreased incidence of PD among individuals who had been recorded to take DPP-4 inhibitors (276). This is in contrast to the UK based studies (NCT01971242 and NCT01971242) which noted a positive and persistent effect of Exenatide treatment in off-medication motor scores (267).

Given the promising results from clinical trials, an understanding the mechanisms underlying GLP-1 mimetic actions on normal and diseased neural tissue would be invaluable. Neurodegenerative diseases share several pathological features, including but not limited to, synaptic loss and failure, reduced neurogenesis, enhanced free radical production, and cell death [reviewed in (279–282)]. The accumulation of misfolded proteins, common in neurodegeneration, impairs cellular communication and function, and causes the activation of neuronal inflammatory responses by activation of glial cells (microglia and astrocytes). Although such neuroinflammatory responses initially maintain homeostasis, chronic activation leads to increased severity of the disease state (283–285). Fortunately, GLP-1 effects in the brain are reminiscent of its actions on pancreatic β-cells, signaling through GLP-1R to initiate anti-inflammatory, anti-apoptotic and pro-survival effects (250, 286, 287).

The pro-survival effects of GLP-1 on neurons are attributable to the reduction of ER-stress and enhanced autophagy in neural cells. Studies by Panagaki et al. (288) as well as Chen et al. (289) reported that liraglutide enhanced AKT signaling and STAT3 activation, resulting in reduced apoptosis. These effects can also arise from activation of GLP-1R in astrocytes and microglia (290), which when triggered can reduce the levels of pro-inflammatory cytokines such as TNF-α and IL-1β in different models of brain inflammation (290–293). In PD, chronic activation of microglia can trigger polarization toward the cytotoxic M1 macrophages, leading to a self–perpetuating persistent inflammatory environment (294, 295), considered to be a major factor in driving dopaminergic degeneration (267, 296, 297). Use of Exenetide has been reported to halt dopaminergic degeneration and restore dopamine (DA) imbalance induced by 6-OHDA, MPTP, and Lipopolysaccharide in animal toxin models (271, 287, 298). Although mechanism of action through which GLP-1 stimulates microglial function in regards to chronic inflammation remain unclear, several studies point to NF-κB activation achieved through DPP-IV inhibitors in

rotenone induced rodent PD models. Increased levels of NF-κB are observed in tyrosine hydroxylase (TH) and dopaminergic neurons, astrocytes and microglia, with implications on the pathogenesis of PD; while inhibition of NF-κB is correlated with neuroprotective effects in such PD models (299, 300). Since GLP-1 signaling activates AKT, one plausible explanation for its therapeutic effect in PD to reduce glial inflammation is through this increased activity in order to elevate levels of the Inhibitor of NF-κB (IκBα), ultimately leading to a reduction in neuroinflammation (26, 301).

Extending from these findings, GLP-1 mimetics can also act upstream of chronic neuronal inflammatory responses in cells by ameliorating the accumulation of misfolded proteins. The accumulation of misfolded proteins can occur through dysregulation of key cellular process, which in turn adversely affects neuronal homeostasis (267, 302–305). GSK-3 α/β isoforms are an example of constitutively active key regulatory enzymes, which when recruited and activated, by α-Synuclein (α-Syn) (a key mediator of PD), leads to hyperphosphorylation of Tau and subsequent increased accumulation of amyloid aggregates (306–309). Several *in vivo* and *in vitro* studies have showed that GLP-1 administration can protect neurons from Aβ aggregation, advanced glycation end products (AGEs) insult, and reduce tau hyperphosphorylation through regulation of GSK-3β signaling. The proposed mechanism of action of GLP-1 is believed to occur through activation the PI3K/AKT signaling pathway, leading to phosphorylation and deactivation of GSK-3β amino-terminal serine residue (271, 302, 310, 311). As previously indicated, GLP-1R activation may reduce protein aggregation through autophagic clearance, however a study by Yuan et al. (312) in 2015, indicated that rotenone induced alterations to autophagy and α-Syn clearance, are mediated by Ca^{2+}/AKT/GSK-3β signaling pathway. Particularly, the authors reported that rotenone treatment of PC12 cells (derived from a tumor of the rat adrenal medulla) increased intra-cellular Ca^{2+} which, in turn, induce aggregation and phosphorylation of α-Syn and impair autophagy. While the hypothesis that raised intracellular Ca^{2+} promotes aggregation α-Syn is supported by previous work (313), the claim that rotenone induced alterations to autophagy and α-Syn, are mediated by Ca^{2+}/GSK-3β signaling pathway may be an oversimplified statement, but nonetheless, reveals an additional mechanism by which GLP-1R signaling can alleviate protein aggregation. As Ca^{2+} is a second messenger in the cell, inappropriate fluctuations would undoubtedly impact autophagy and protein aggregation, and through the use of rotenone, an inhibitor of mitochondrial complex I, leads to elevation of intracellular Ca^{2+} through inhibition of resting background K^{+} currents, membrane depolarization, and VDCC opening, This would in turn impact ATP production, inhibit AC activity, down regulate cAMP signaling, and disrupt mitochondrial membrane potential (314, 315). Changes in cellular ATP, and mitochondrial stability, induced by protein aggregation would not only promote apoptosis, but increase cellular ROS, and oxidative stress, all of which act together to contribute to the destabilization of ER homeostasis and autophagy in neurodegenerative diseases (314, 316–319). GLP-1R activation can act to mitigate the deleterious effects of overloaded intracellular Ca^{2+}, as mentioned before, through the cAMP/PKA/EPAC pathways and is thought to be an integral mechanism in the prevention of spatial memory and hippocampal synaptic plasticity impairments arising from Aβ-induced toxicity (43, 320, 321). The GLP-1 mediated regulation of Ca^{2+} is also coupled to restoration of insulin signaling throughout the brain, which can further promote its pro-survival abilities. This is crucial as impaired insulin signaling in AD and PD patients has been reported to negatively impact dendritic sprouting, neuronal stem cell growth and tissue repair (322–324).

Within the brain, endogenous, brain derived GLP-1 can promote insulin release (325, 326), thereby potentially increasing the expression of the internalized IR and IGF-1R in AD patients. Such a mechanism has recently been reported to restore IR signaling deficits through decreases of the c-Jun N-terminal kinase (JNK) signaling in symptomatic (?) T2D mice (326). These studies are furthered in rodent models whereby both chronic and acute treatments of liraglutide have been shown to protect against Aβ-induced impairment of memory and spatial learning in rats (315), as well as prevent memory impairment, reduce β-amyloid plaque, and plaque induced chronic inflammation in the APP/PS1 AD mice model (315, 327). AD rodent models chronically treated with the GLP-1 analog Val(8)glucagon-like peptide-1 caused modulation of neurotransmitter release, synaptic transmission (LTP) formation, and restoration of synaptic plasticity, as well as preventing impairment in the learning of new spatial tasks (328–330). Restoration of IR signaling, acts synergistically with GLP-1 signaling, modulating autophagy, oxidative stress, protein synthesis, apoptosis, and mitochondrial biogenesis (331, 332). GLP-1's oxidative stress protective mechanisms are indicated to be ameliorated in primary cortical neurons by GLP-1/CREB signaling inducing expression of apurinic/apryimidinic endonuclease 1 (APE1), a key enzyme of the base excision DNA repair (BER) pathway (333), while also impacting protein aggregation induced neurotoxicity through enhanced mitochondrial function. This has been indicated to occur through the deacetylase SIRT1 (334), its promotion of heat shock protein 70 (HSP70), an augmenter of normal α-Sync folding (335), modulation of PGC-1α, as well as activation of ADAM10, through retinoic acid receptor β, leading to the reduction of plaque formation (336).

It is evident that the mechanism through which GLP-1 stimulates pro-survival signaling, reduces neural tissue inflammation and improves cognitive function is complex. However, by defining cell-type specific signaling pathways in neural cells, it may be possible to develop distinct treatment strategies that uniquely modulate GLP-1 signaling in mental illness as well as neurodegenerative diseases.

CONCLUSIONS

GLP-1 promotes glycemic control through a plethora of widely recognized physiological mechanisms. Among them, stimulation of insulin secretion and inhibition of glucagon release directly improve postprandial glucose homeostasis, while inhibition of gastric emptying and food intake represent a longer term positive

effect of limiting weight gain. Due to these properties, GLP-1 therapies have been routinely and successfully used for the treatment of T2D and obesity for more than a decade. Most recent studies unveiled that GLP-1 analogs also act in the CNS and various peripheral tissues to restore and maintain normal cellular functions. This has been demonstrated in response to a variety of distinct disease paradigms and physiological insults, either through direct cell autonomous effects or through indirect whole body metabolic improvements. In this review, we provided a thorough description of the diverse roles for GLP-1R signaling across multiple tissues, focusing in the downstream pathways stimulated by acute and chronic activation of the receptor, and discussed novel pleiotropic applications of GLP-1 mimetics in the treatment of human disease. Continuing efforts to delineate tissue specific mechanisms of GLP-1 action are necessary in order to identify novel translational alternatives and foster the development of new GLP-1-based therapeutic agents harnessing different aspects of GLP-1 biology with therapeutic potential not only for T2D and obesity, but also for heart, liver, kidney, lung and brain related disorders.

AUTHOR CONTRIBUTIONS

JR and RC conceived the idea, organized, and designed the manuscript. JR wrote the first draft, which was reviewed by RC, JH, and PN. Figures were designed by JR and RC. All authors contributed in manuscript revision and agreed with the final submitted version.

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REVIEW
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Future Perspectives on GLP-1 Receptor Agonists and GLP-1/glucagon Receptor Co-agonists in the Treatment of NAFLD

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Along the obesity pandemic, the prevalence of non-alcoholic fatty liver disease (NAFLD), often regarded as the hepatic manifestation of the metabolic syndrome, increases worldwide representing now the prevalent liver disease in western countries. No pharmacotherapy is approved for the treatment of NAFLD and, currently, the cornerstone treatment is lifestyle modifications focusing on bodyweight loss, notoriously difficult to obtain and even more difficult to maintain. Thus, novel therapeutic approaches are highly demanded. Glucagon-like peptide-1 (GLP-1) receptor agonists (GLP-1RAs) are approved for the treatment of type 2 diabetes and obesity. They exert their body weight-lowering effect by reducing satiety and food intake. GLP-1RAs have also been shown to reduce liver inflammation and fibrosis. Furthermore, glucagon receptor agonism is being investigated for the treatment of NAFLD due to its appetite and food intake-reducing effects, as well as its ability to increase lipid oxidation and thermogenesis. Recent studies suggest that glucagon receptor signaling is disrupted in NAFLD, indicating that supra-physiological glucagon receptor agonism might represent a new NAFLD treatment target. The present review provides (1) an overview in the pathophysiology of NAFLD, including the potential involvement of GLP-1 and glucagon, (2) an introduction to the currently available GLP-1RAs and (3) outlines the potential of emerging GLP-1RAs and GLP-1/glucagon receptor co-agonists in the treatment of NAFLD.

Keywords: glucagon-like peptide-1, glucagon-like peptide-1 receptor agonist, glucagon, non-alcoholic fatty liver disease, non-alcoholic steatohepatitis

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is defined as fat accumulation in more than 5% of the hepatocytes. NAFLD can be subdivided according to the level of inflammation ranging from simple steatosis without inflammation to non-alcoholic steatohepatitis (NASH), which is often associated with fibrosis and over time may lead to cirrhosis and end-stage liver failure. NAFLD also increases the risk of hepatocellular carcinoma (HCC) (1).

The prevalence of NAFLD is increasing. Nearly 25% of the population in western countries have NAFLD and up to 6.5% fulfill the criteria of NASH (2). The increased prevalence of end-stage liver failure and HCC due to progressive NAFLD has led NAFLD to become the second most common indication for liver transplantation, likely configuring the leading indication for liver transplantation within the next two decades (3).

NAFLD is considered the "hepatic manifestation" of metabolic syndrome (4). Indeed, NAFLD is associated with central visceral adiposity except for a small proportion of lean patients, in whom genetic predisposition might play a crucial role in liver steatosis and fibrosis. Most morbidly obese patients undergoing bariatric surgery have NAFLD, nearly 30% have NASH, and 10% have advanced liver fibrosis (5). NAFLD is also closely linked with type 2 diabetes (T2D). In T2D the prevalence of NAFLD raises up to 70–75%, and the prevalence of NASH and advanced fibrosis are 65 and 15%, respectively (6). Importantly, coexisting T2D almost doubles the rate of which NAFLD progresses to end-stage liver disease and HCC, respectively (7, 8). A recent meta-analysis comprising nearly 300,000 individuals showed that patients with NAFLD have an increased risk of developing T2D compared to controls [hazard ratio (HR) 2.22, 95% CI 1.84–2.60], and that risk of T2D increases across the stages of NAFLD (9). In addition to T2D, NAFLD is accompanied and complicated by several other extra-hepatic manifestations. By stimulating pro-inflammatory and pro-thrombotic factors, it contributes to the development of several chronic diseases, including ischemic heart disease, cardiomyopathy, cardiac arrhythmias and chronic kidney disease. Noteworthy, the leading cause of mortality in NAFLD is cardiovascular disease (CVD) (10, 11).

No pharmacological therapies are approved for the treatment of NAFLD and lifestyle changes focusing on caloric restriction and weight loss constitute the general treatment recommendations. Recent trials investigating glucagon-like peptide-1 (GLP-1) receptor (GLP-1R) agonists (GLP-1RAs) for the treatment of NAFLD have shown promising results. Furthermore, GLP-1R/glucagon receptor dual agonists are being investigated for the treatment of NAFLD (12). In addition to its effects on glucose metabolism, glucagon is suggested to induce body weight loss, by increasing satiety and enhancing hepatic lipid oxidation and whole-body energy expenditure (13). This review provides (1) insights into the pathogenesis of NAFLD including the potential involvement of GLP-1 and glucagon, (2) a critical appraisal of the applicability of GLP-1RAs in NAFLD treatment, and (3) a review of the evidence for GLP-1/glucagon receptor co-agonism as a novel approach in the treatment of NAFLD.

THE PATHOGENESIS OF NON-ALCOHOLIC FATTY LIVER DISEASE

The first phase of NAFLD is characterized by accumulation of fat in the liver (hepatic steatosis), which may progressively lead to NASH (in 5–20% of patients) with or without concomitant fibrosis. Among the patients who develop NASH, 10–20% will progress to higher-grade fibrosis and approximately 5% will develop overt cirrhosis (14). It is arguable whether advanced fibrosis may regress, whereas steatosis and NASH are both reversible conditions (15). NAFLD-associated cirrhosis has traditionally been regarded as the leading risk factor for the development of HCC. However, HCC may also occur in a non-cirrhotic liver (16, 17). This suggests that NAFLD might not necessarily implicate a sequential process to evolve (**Figure 1**) (18).

Triglycerides (TG) accumulation is likely one of the first steps in the pathophysiology of NAFLD as a result of an impaired free fatty acid (FFA) metabolism in the liver (**Figure 2**). Excessive caloric intake increases FFA load to the liver to a point that the ability of the hepatocytes to oxidize FFA or re-esterify to TG and secrete very low-density lipoproteins (VLDL) is overwhelmed. Thus, TG accumulate in forms of lipid drops (steatosis). Moreover, insulin resistance of the adipose tissue, associated with overweight/obesity, contributes to the flux of FFA from adipose tissue to the liver through unrestricted lipolysis (19). Lastly, increased *de novo* lipogenesis, i.e., hepatic FFA synthesis, seems to contribute to lipid deposition (20). Prolonged accumulation of lipids in hepatocytes is associated with lipotoxicity, which may initiate inflammation, apoptosis and ultimately fibrosis (21). The main route of hepatic fat oxidation is the mitochondrial tricarboxylic acid (TCA) cycle. An overactive TCA cycle stresses the endoplasmic reticulum, thus inducing mitochondrial dysfunction and formation of reactive oxidative species and toxic lipid intermediates, like ceramides and diacylglycerol (22, 23). Insulin resistant adipose tissue may also enhance inflammation by lowering release of anti-inflammatory adipokines such as adiponectin and increasing release of leptin and pro-inflammatory cytokines like interleukin 6 (IL-6) and tumor necrosis factor-alpha (TNF-α) (24). This inflammatory milieu may contribute to hepatic insulin resistance and thus establish a vicious circle. At a molecular level, serine phosphorylation of insulin receptor substrate-1 (IRS-1) by inflammatory signals appears to be one of the key aspects that disrupt insulin-receptor signaling (25).

The gut-derived incretin hormones GLP-1 and glucose-dependent insulinotropic polypeptide (GIP) are responsible for the so-called incretin effect (i.e., the potentiation of glucose-stimulated insulin secretion after meal ingestion) (26). Additionally, GLP-1 suppresses glucagon release from pancreatic alpha cells, delays gastric emptying and enhances satiety (27). While GIP displays similar insulinotropic properties, it has been shown to act as a bifunctional blood glucose stabilizer

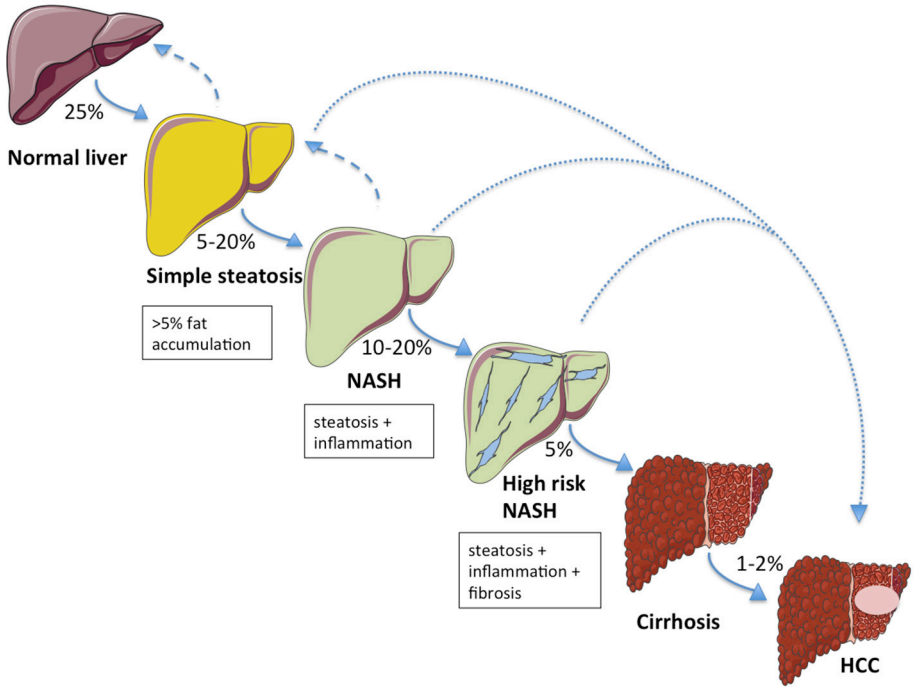

FIGURE 1 | The NAFLD spectrum and relative probabilities to progress across the stages of liver damage. Aside to a classical development in the natural history of the disease, alternative routes (dotted lines) directly leading to hepatocellular carcinoma (HCC) from simple steatosis or NASH are possible. Hepatic steatosis and NASH are both reversible conditions (dashed arrows).

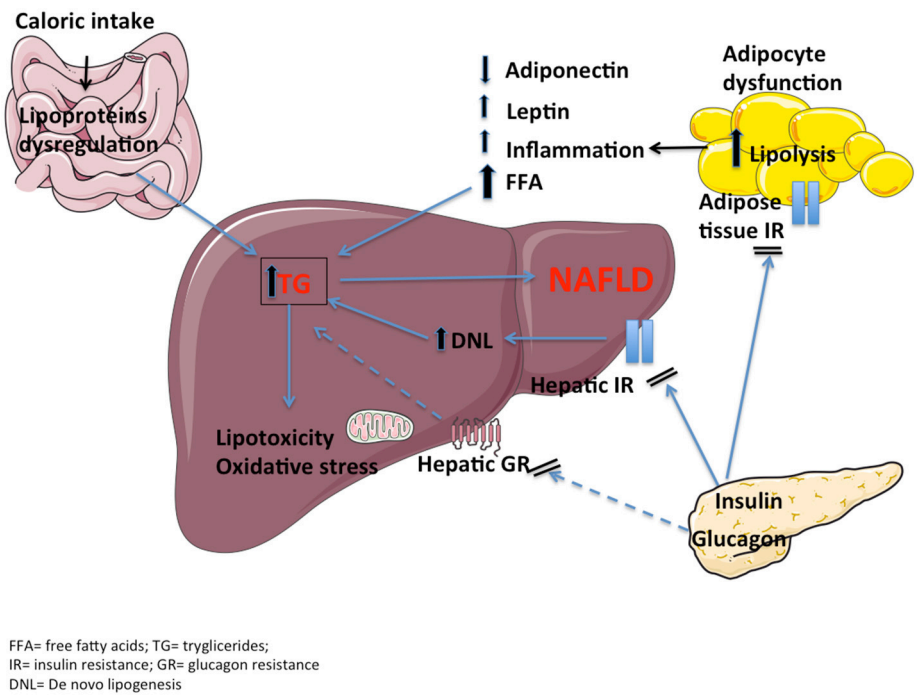

FIGURE 2 | The pathophysiology of NAFLD includes dietary fat contribution, hepatic and adipose tissue insulin resistance, proinflammatory cytokines, lipotoxicity and oxidative stress. A reduced hepatic glucagon resistance (dashed lines), together with an impaired incretin effect, may be additional mechanisms.

by stimulating glucagon release in the presence of low plasma glucose levels. Moreover, GIP receptor activation has reported contrasting effects on satiety, caloric intake and body weight (28). It has been suggested that patients with NAFLD have lower concentrations of biologically active incretin hormones compared to healthy individuals, which may be a consequence of an increased degradation by dipeptidyl peptidase 4 (DPP-4) (the enzyme, which under normal conditions inactivates the incretin hormones) (29) or a decreased production (30, 31). Conversely, a series of studies by our group suggest that patients with NAFLD have normal GLP-1 and GIP plasma levels, even though they exhibit a reduced incretin effect (32). Whether a reduced incretin effect (reduced beta cell sensitivity to GIP and/or GLP-1) may play a role in the pathophysiology of NAFLD warrants further investigations.

Glucagon is a key hormone in the regulation of overall energy homeostasis during the fasting state and other energy-demanding situations. Beyond the stimulation of hepatic glucose production, it also affects hepatic fat metabolism promoting lipid oxidation and lowering lipid synthesis. Glucagon decreases food intake and appetite by central mechanism and by reducing gastric emptying (33, 34). Furthermore, glucagon may display thermogenic properties, inducing an increase in energy expenditure through brown adipose tissue activation (13, 35). It has been hypothesized that hepatic glucagon resistance might play an important role in fat accumulation in the liver and *vice versa* (36). Preclinical studies in NAFLD have demonstrated that a reduction in G protein-coupled glucagon receptor (GCGR) signaling results in an increase of hepatic fat content (37, 38). Moreover, a recent study from *Guzman* and colleagues (39) has shown that, in patients with T2D, treatment with a selective GCGR antagonist, LY2409021, induces a significant increase in hepatic lipid content assessed by magnetic resonance imaging, suggesting that GCGR activation is required to prevent build-up of fat in the hepatocytes. It has been hypothesized that a reduction in hepatic GCGR and signaling molecules affects a feedback mechanism acting on the pancreatic alpha cells, increasing glucagon secretion, and this liver-pancreas axis might contribute to fasting hyperglucagonemia (36). In line with this hypothesis, results from our group show that individuals with NAFLD (both normoglycemic individuals and patients with T2D) exhibit significantly higher fasting plasma glucagon levels compared to matched controls without NAFLD (28). However, whether hyperglucagonemia is directly involved in the pathogenesis of NAFLD or is a consequence of steatosis remains uncertain.

TREATMENT OF NAFLD: APPLICABILITY OF GLP-1RAs

Potential Modes of Action of GLP-1RAs in NAFLD

Current NAFLD treatment consists of interventions promoting bodyweight loss. It has been estimated by studies with ^{1}H-magnetic resonance spectroscopy that decreasing bodyweight by 10% via diet combined with physical activity can induce a reduction in hepatic TG concentration up to nearly 60% in overweight individuals (40). Bariatric surgery is the most effective treatment in severely obese patients, inducing significant improvement in lobular inflammation and a disappearance of NASH in 50–85% of cases (41). Currently no pharmacological treatment has proven efficacious, however numerous drugs targeting key-steps in NAFLD pathogenesis are under investigation. These compounds can be grouped in medications targeting (1) metabolic derangements including excess bodyweight, (2) inflammation and oxidative stress, and (3) dysregulation of the gut-liver axis (42). In this regard GLP-1RAs exhibit potent metabolic effects, however they might also affect other of the proposed targets. In the following paragraphs, we will present the potential mechanisms of action of GLP-1RAs in NAFLD provided by studies in humans (**Figure 3**).

Metabolic Effects

Bodyweight reduction

GLP-1 has a documented dose-dependent effect on satiety, through central mechanisms in the hypothalamus and brainstem. Accordingly, a reduced caloric intake has been observed in lean and obese individuals and in patients with T2D after exogenously administered GLP-1 during *ad libitum* meals. In addition, weight loss is a consistent finding in clinical trials investigating GLP-1RAs (27).

Reduction of hepatic and adipose tissue insulin resistance

An improved insulin sensitivity is expected after chronic treatment with GLP-RAs mainly due to marked bodyweight reductions. However, this effect might be independent of changes in visceral fat accumulation since previous studies have demonstrated that the hepatic glucose production is decreased in healthy individuals following acute administration of both native GLP-1 (43) and the GLP-1RA exenatide (44). Furthermore, in patients with NASH, GLP-1 decreases *de novo* lipogenesis and reduces levels of lipolysis-induced FFA and triglyceride-derived toxic metabolites (45). Whether these actions could be partly mediated through hepatic GLP-1R signaling remains uncertain, as the presence of GLP-1Rs in the liver has not been confirmed (46–48).

Insulinotropic effect

As alluded above, patients with NAFLD, in whole its spectrum including cirrhotic individuals, show a reduced incretin effect. Whether the insulinotropic action of GLP-1RAs, overcoming the reduced incretin effect, might ameliorate NAFLD is however still uncertain.

Inflammation and Oxidative Stress

The impact of GLP-1RAs on hepatic lipotoxicity has been extensively explored in cellular and animal models, whereas few clinical studies have been conducted (49). In NAFLD patients, the increase in serum concentrations of total adiponectin following GLP-1RAs treatment may be consistent with a restoration of a dysfunctional adipose tissue (50). Liraglutide also decreases fasting serum leptin resulting in a significant reduction in the leptin-to-adiponectin ratio (45). In turn, adiponectin can ameliorate NAFLD-associated liver abnormalities by regulating

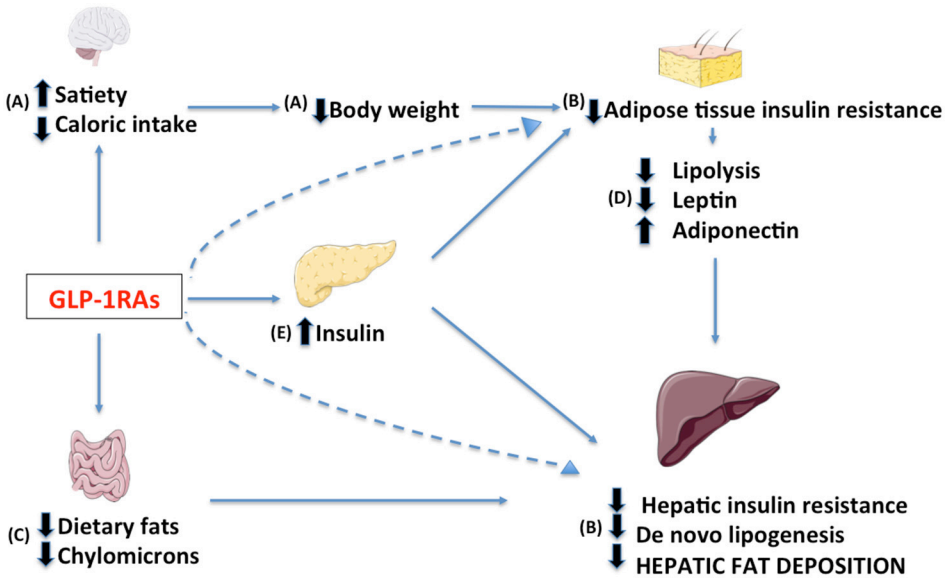

FIGURE 3 | Potential targets of GLP-1 receptor agonists in the treatment of NAFLD include: **(A)** a decrease in caloric intake through central regulation of satiety, **(B)** a reduction of hepatic and adipose tissue insulin resistance due to decrease in body weight, (a direct effect may not be excluded, dashed lines) **(C)** a modified intestinal lipoprotein metabolism, **(D)** a resolution of dysfunctional adipose tissue and **(E)** an enhancement of insulin release.

the oxidation of hepatic fatty acid and the activity of acetyl-CoA carboxylase and fatty acid synthase, two key enzymes involved in fatty acid synthesis (51).

Gut-Liver Axis

Lipoproteins production by the liver and by the intestine is subject to a variety of hormonal and nutritional modulators and is deranged in T2D as well as in insulin resistant states including NAFLD (52). As carefully reviewed by Xiao et al. numerous studies have demonstrated that GLP-1RAs may ameliorate postprandial lipidemia during meal tests by multiple pathways including decreased absorption of dietary fats as consequence of reduced gut motility and direct inhibition of chylomicron synthesis and secretion (53).

GLP-1RAs and Clinical Trials in NAFLD

Lixisenatide

In a systematic meta-analysis including 12 randomized controlled trials (RCTs) comparing lixisenatide to placebo or active interventions in T2D, lixisenatide was reported to normalize levels of alanine aminotransferase (ALT) in a greater proportion of overweight and obese patients with T2D than comparators (54). However, at present, no trial aimed at testing the efficacy of lixisenatide in patients with NAFLD has been conducted.

Exenatide

Almost all human studies with exenatide twice-daily evaluating NAFLD-related endpoints involve patients with T2D. Several case series (55, 56) and open-label trials (57–60) suggest that the combination of a better glycemic control, improved metabolic parameters and bodyweight reductions achieved by exenatide treatment as monotherapy or as add-on to standard therapies may lead to improvements in liver biomarkers and hepatic fat reductions in patients with T2D. However, whether exenatide is able to ameliorate histological features of NAFLD/NASH has not been investigated by RCTs.

Liraglutide

Liraglutide is the only GLP-1RA, which has been investigated for the treatment of NAFLD. In the "Liraglutide Efficacy and Action in Diabetes" (LEAD) programme (61), a total of 154 patients with T2D within the LEAD-2 trial participated in a sub-study to assess liver fat content by the liver-to-spleen attenuation ratio at a computer tomography (CT) scan. Such ratio significantly increased from baseline after 26 weeks of treatment with liraglutide 1.8 mg/day, indicating a reduction in liver steatosis, whereas it was unchanged in patients treated with lower doses of liraglutide, glimepiride or placebo. Liraglutide was also associated with a reduction in mean ALT levels, which, however, disappeared after correction for changes in weight and HbA_{1c} (62, 63). An open-label uncontrolled trial including 27 patients with T2D and NAFLD treated with liraglutide 0.9 mg/day for 24 weeks showed a trend toward increases in liver-to-spleen attenuation ratio assessed by CT scan and, more importantly, a significant improvement in histological inflammatory scores in 10 subjects undergoing liver biopsies after a prolonged treatment of 96 weeks (64). In patients with poorly controlled T2D, 6 months of treatment with liraglutide 1.2 mg/day significantly reduced liver fat content as evaluated by ^{1}H-magnetic resonance spectroscopy, an effect mainly driven by bodyweight loss (65). The first RCT to

investigate the effect of liraglutide in patients with NAFLD was the LEAN study, in which 52 patients with NASH were randomized to either 48 weeks of treatment with liraglutide (1.8 mg/day) or placebo (66). In this study, 39% of the patients treated with liraglutide achieved histologic resolution of NASH as compared to 9% in the control group ($p = 0.019$). Furthermore, worsening of fibrosis was significantly reduced with liraglutide compared to placebo. With respect to the previously mentioned study, this proof-of concept study has important methodological strengths residing in the availability of baseline and post-treatment hepatic biopsies. Interestingly, reductions in bodyweight and $HbA1_c$ were similar in patients with improvements in liver histology ("responders") and those without ("non-responders"), suggesting that, beyond bodyweight reduction and improvement in glucose control, other mechanisms may be involved in the beneficial effects of liraglutide. The effect of liraglutide in NAFLD has also been compared to other antidiabetic drugs. In 87 patients with NAFLD and T2D, 12-week treatment with liraglutide 1.8 mg/d resulted in similar reductions in intra-hepatic fat as treatment with metformin, whereas liraglutide was significantly more effective than gliclazide. In this trial, no differences in bodyweight changes or glucose control among treatments were observed (67). In one study, 12-week treatment with insulin glargine *vs* liraglutide (mean dose = 1.3 mg/day) showed similar glycemic control and decrease of hepatic fat burden, although a reduction in BMI was only observed in the liraglutide-treated patients (68). Furthermore, in a 12-week RCT in which patients were either assigned to liraglutide (1.8 mg/day), sitagliptin (100 mg/day) or placebo, no difference in glycemic control or bodyweight reductions were found, and both treatments failed to reduce liver hepatic fat content compared to placebo (69). A recent randomized study comparing structured lifestyle modification (a cornerstone in the currently recommended treatment of NASH) to liraglutide 3 mg/day without lifestyle modifications showed similar reductions in ALT and liver stiffness with no difference in bodyweight variations. This findings indicate that an additive effect of lifestyle modifications and liraglutide 3 mg/day might exist (70).

Semaglutide

An ongoing phase 2 trial (NCT02970942) is currently investigating the efficacy and safety of three doses of subcutaneous semaglutide once-daily versus placebo in subjects with NASH. It will include 288 participants. As in the LEAN study, the primary outcome is histologic resolution of NASH without worsening of fibrosis after 72 weeks of treatment, while secondary outcomes include improvement in liver fibrosis ($\geq$1 stage) with no worsening of NASH, NAFLD activity score, as well as multiple serum markers of fibrosis. Furthermore, a RCT (NCT03357380) is currently comparing the change in early stages of scar tissue as well as fat deposition in the liver, as detected by magnetic resonance imaging scans, in patients treated with semaglutide or placebo for 72 weeks.

An overview on the clinical trials investigating GLP-1RAs treatment in NAFLD is presented in **Table 1**.

EMERGING GLP-1 AND GLUCAGON RECEPTOR CO-AGONISTS

Multiple GLP-1R co-agonists are emerging for the treatment of obesity and diabetes. These agents, comprising GLP-1 combined molecules with glucagon or other hormones, have been investigated in preclinical studies for NAFLD treatment. Combination therapy and hybrid molecules that act through multiple receptors appear to maximize the beneficial outcomes, without increasing side effects of the single molecules. GLP-1 and glucagon display similar amino acid N-terminal sequences and bind to structurally related receptors, facilitating the development of single-molecule GLP-1R/GCGR co-agonists. In general, GLP-1 and glucagon are believed to antagonize their respective effects on glucose homeostasis. Whereas GLP-1 decreases plasma glucose levels by exerting insulinotropic effects, glucagon stimulates hyperglycemia by enhancing hepatic glucose output. However, novel dual receptor agonists have been developed for the treatment of obesity and T2D under the concept that GLP-1 restrains the hyperglycemic action of glucagon, while allowing it to exert its beneficial actions on bodyweight, food intake, lipid metabolism and thermogenesis (71).

One month-therapy with a pegylated GLP-1R/GCGR dual agonists in diet-induced obese (DIO) mice resulted in bodyweight loss and improved glycemic control. These effects were coupled to an amelioration in lipid metabolism and hepatic steatosis, which markedly exceeded the effect of single GLP-1RA treatment (72). In 2016, another balanced dual receptor agonist demonstrated pronounced effect on bodyweight and glucose control, together with reducing hepatic fat content in rodents and non-human primates (73). Recently, chronic exposure of a GLP-1/glucagon dual analog conjugated with maleimide showed beneficial effects on liver morphology in DIO mice (74).

Oxyntomodulin, a gut-derived peptide hormone, activates both the GLP-1R and the GCGR, although, with reduced affinity compared to GLP-1 and glucagon, respectively. Oxyntomodulin has already shown to reduce food intake and bodyweight in rodents and humans (75, 76). Interestingly, in a mouse model of NASH, 2 weeks of treatment with a oxyntomodulin analog also ameliorated the hepatic histopathological features of this disease (77).

In addition to GLP-1R/GCGR dual agonists, a chimeric peptide as dual GLP-1/GIP receptor agonists has been developed, showing enhanced therapeutic potential for obesity and related comorbidities. When compared to single agonists, unimolecular dual incretin was more effective in correcting adiposity-induced insulin resistance in animal models of obesity and diabetes; it also improved liver function by reversing hepatic steatosis features in histopathological specimens of DIO mice. In healthy and diabetic subjects, the co-agonist displayed to improve glucose tolerance and insulin secretion, although no data regarding bodyweight, lipid metabolism, or liver function were reported (78). However, preliminary studies in non-diabetic obese individuals showed that simultaneous activation of GLP-1 and GIP receptors did not potentiate GLP-1-mediated effects in lowering food intake and appetite (79).

TABLE 1 | Clinical trials with GLP-1RAs in NAFLD.

Subjects (number)	Study type	Diagnostic test	Intervention (dose)	Comparator (dose)	Duration (weeks)	Outcome evaluation	Effect	References
T2D (8)	Case series	Biopsy-proven NAFLD/NASH	Exe (10 μgx2/d)	–	28	Liver biopsy	**(+)** histological inflammation and fibrosis	(55)
T2D overweight/obese (974)	Single arm-trial	Liver enzymes	Exe (10 μgx2/d)	–	104	Liver enzymes	+ 39% of subjects with elevated baseline ALT had normalization of ALT	(57)
T2D obese (125)	Open-label	FLI	Exe (10 μx2/d alone or add-on Met and/or SU)	Met and/or SU	26	FLI	+ improvement of FLI in Exe group vs. oral antidiabetic agents	(58)
T2D overweight/obese (60)	RCT	Liver enzymes; Ultrasound	Exe (10 μgx2/d)	Intensive treatment with insulin glargine	12	Liver enzymes; Ultrasound	+ reduction of liver enzymes and degree of fatty liver at ultrasound with Exe vs. Glargine	(59)
T2D overweight (117)	RCT	Liver enzymes	Exe (10 μgx2/d)	Met	12	Liver enzymes	+ reduction of liver enzymes	(60)
T2D overweight/obese (27)	Single arm-trial	Biopsy-proven NAFLD/NASH	Lira (0.9 mg/d)	–	24 96 (*N* = 10)	CT scan Liver biopsy (*N* = 10)	**(+)** liver to spleen attenuation ratio + histological inflammation and fibrosis	(64)
T2D overweight/obese (68)	Single arm-trial	^{1}H-MR spectroscopy	Lira (1.2 mg/d)	–	26	^{1}H-MR spectroscopy	+ 31% liver fat content	(65)
Overweight/obese (17 T2D out of 52)	RCT	Biopsy-proven NASH	Lira (1.8 mg/d)	Placebo	48	Liver biopsy	++ 39% histological resolution of NASH with Lira vs. 9% with Placebo	(66)
T2D (154)	RCT (LEAD-2 substudy)	CT scan	Lira (1.8-1.2-0.6 mg/d) add on-Met	Glimepiride (4 mg) or Placebo add on- Met	26	CT scan	+ 10% liver to spleen attenuation ratio with Lira 1.8 mg/day vs. no effect with other treatments	(62)
T2D (87)	RCT	Ultrasound	Lira (1.8 mg/d)	Met or Gliclazide	24	Ultrasound	+ improvement in hepatic/renal ratio index in Lira vs. Gliclazide	(67)
T2D overweight/obese (35)	RCT	^{1}H-MR spectroscopy; MR imaging	Lira (0.6 to 1.8 mg/d)	Insulin glargine titrated to achieve FPG<7mM	12	^{1}H-MR spectroscopy and MR imaging	±no difference in liver fat reduction between Lira and Glargine	(68)
T2D overweight/obese (52)	RCT	^{1}H-MR spectroscopy	Lira (1.8 mg/d)	Sitagliptin (100 mg) Placebo	12	^{1}H-MR spectroscopy	± no effect in liver fat content with Lira, as well as Sitagliptin or Placebo	(69)
NonT2D obese	RCT	MR imaging	Lira (3 mg/d)	Intensive lifestyle intervention	26	MR imaging	± no difference in liver fat reduction between Lira and lifestyle intervention	(70)
288 patients with T2D (288)	RCT	Biopsy-proven NASH	Sema (0.1-0.2-0.4 mg/d)	Placebo	72	Liver biopsy	Ongoing (NCT02970942)	
T2D (66)	RCT	MR imaging	Sema (0.4 mg/d)	Placebo	72	MR imaging	Ongoing (NCT03357380)	

d, day; FLI, fatty liver index; FPG, fasting plasma glucose; Lira, liraglutide; MR, Magnetic resonance; Met, metformin; NAFLD, Non-alcoholic fatty liver disease; NASH, Non-alcoholic steatohepatitis; Sema, semaglutide; SU, sulphonylurea; T2D, type 2 diabetes; (+), trend improvement in predefined outcome; **+,** *significant improvement in predefined outcome;* **++,** *significant improvement in predefined outcome judged by gold-standard method; ±, no significant effect in predefined outcome.*

This approach with dual agonists has been followed by the development of monomeric triagonists, incorporating residues from GLP-1, glucagon and GIP. In high-fat diet fed mice, treatment with the triagonist dose-dependently improved steatohepatitis and reduced levels of ALT, underpinning the potential for this compound to treat liver disease (80). While multi-agonism with incretin hormones and glucagon has demonstrated great therapeutic potential, the conceptual approach of polypharmacology has also been extended to other hormone combinations. In pre-clinical studies, GLP-1 mediated delivery of estrogen or dexamethasone has proven beneficial effects on glucose tolerance, bodyweight control and systemic inflammation (81). Interestingly, a conjugated glucagon and thyroid hormone (T_3) agonist has shown to reverse metabolic syndrome related abnormalities (82). In a rodent model of NASH, 3-week treatment with glucagon/T_3 lowered ALT levels and improved macroscopic and histological features of NASH, including reversal of fibrosis (82).

CONCLUSION AND FUTURE PERSPECTIVES

New knowledge about the pathophysiology of NAFLD has been accumulating over the last decade, displaying the complexity of the mechanisms involved in the development of this condition. In addition to bodyweight loss through lifestyle interventions, pharmacotherapies targeting adipose tissue, the digestive system (gut-liver axis) and/or inflammation are warranted. In this perspective, GLP-1RAs may act through all of these different pathways. However, most of the available GLP-1RAs have still not been thoroughly investigated for the indication of NAFLD. Liraglutide treatment has been shown to improve NASH histology and reduce progression of fibrosis (66). Clinical trials investigating the newly approved GLP-1RA semaglutide for the treatment of NASH are currently ongoing. Another approach, which seems promising for future treatment of NAFLD, is the combination of GLP-1 and glucagon, since the latter may potentiate incretin-mediated weight loss and increase lipid utilization and FFA oxidation in the liver. With the additional development of multiple new dual- and tri-agonist, GLP-1 and glucagon-based poly-agonists in the treatment of NAFLD represent an exciting novel pharmacological approach. Whether the promising preclinical pharmacology will result in successful clinical trials is a question that will be answered in coming years.

AUTHORS CONTRIBUTIONS

MS researched the data, made substantial contributions to the discussion of the content, wrote the first draft and reviewed/edited the manuscript. AC, AA, AS, FK, and TV made substantial contributions to the discussion of the content and reviewed/edited the manuscript.

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REVIEW
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Dissecting the Physiology and Pathophysiology of Glucagon-Like Peptide-1

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An aging world population exposed to a sedentary life style is currently plagued by chronic metabolic diseases, such as type-2 diabetes, that are spreading worldwide at an unprecedented rate. One of the most promising pharmacological approaches for the management of type 2 diabetes takes advantage of the peptide hormone glucagon-like peptide-1 (GLP-1) under the form of protease resistant mimetics, and DPP-IV inhibitors. Despite the improved quality of life, long-term treatments with these new classes of drugs are riddled with serious and life-threatening side-effects, with no overall cure of the disease. New evidence is shedding more light over the complex physiology of GLP-1 in health and metabolic diseases. Herein, we discuss the most recent advancements in the biology of gut receptors known to induce the secretion of GLP-1, to bridge the multiple gaps into our understanding of its physiology and pathology.

Keywords: glucagon-like peptide-1, metabolic disease, type 2 diabetes, enteroendocrine cell system, GPCR, L-cells, microbiome, α-cells

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INTRODUCTION

The gastrointestinal (GI) tract is a complex organ that monitors the body's energetical state and provides it with water and macro and micronutrients extracted from the ingested food. Along its length, the enteroendocrine cells (EECs) constitute a complex endocrine organ that communicates with the central nervous system (CNS) and the enteric nervous system (ENS) to orchestrate the homeostatic balance of the body in response to the GI luminal content.

This enteroendocrine system has traditionally been divided into 12 different cell types, based entirely on their hormonal content and cellular morphology. This endocrine organ is not organized in a glandular structure; on the contrary, it is dispersed heterogeneously, mainly as single cells, along the epithelium of the GI tract, from the stomach to the rectum with a defined cephalocaudal, crypt-to-villus in the small intestine and crypt-to-surface distribution in the colon (1, 2).

Despite representing just 1% of the adult gut epithelium, in the last decade it has become clear that the EECs constitute the largest endocrine organ in mammalia (3). Recent analysis of the expression of specific hormones at the cellular level, demonstrated that the EECs subdivision introduced above is outdated. Each enteroendocrine cell co-secretes multiple hormones with spatio-temporal, crypt-to-villus, and rostro-caudal variability, leading to the formation of overlapped gradients of individual hormones along the GI tract; the concept of well-defined subclasses of cells committed to express a specific subset of hormones independent of their location is currently untenable, thus detailed description of the topographical location of the cells needs to be implemented for future clarity (4).

Collectively, the EECs are responsible for the production of more than 30 different hormones that help to orchestrate the fate of the intermediary metabolism; acting upon different organs such as the pancreatic islets, the hypothalamus or the stomach, for the release of insulin, to regulate food intake or gastric emptying respectively (5–8).

Surprisingly, this heterogeneous and highly plastic population of cells is known to differentiate from a single staminal progenitor that gives also rise to enterocytes, goblet and paneth cells (1, 9).

It has been known for more than a century that the gut is capable to stimulate the endocrine portion of the pancreas and even improve the hyperglycaemic state of diabetic patients (10, 11). In 1932, the Belgian investigator LaBarre referred to these "factors" extracted from the intestinal mucosa as "incrétine," deriving it from: INtestinal seCRETion of insulin (12). In the 60s, different authors demonstrated that oral glucose was capable to induce a 2-fold increase in insulin compared to an in-vein isoglycaemic administration (13).

In the last three decades, the incretin-effect has been attributed primarily to two peptide hormones, the gastric-insulinotropic peptide (GIP) and glucagon-like peptide-1 (GLP-1), excreted primarily by duodenal (K) and ileo-colonic (L) enteroendocrine cells respectively (14). Indeed, type 2 diabetes (T2D) is a metabolic disease reported to involve an impaired intestinal release of GLP-1 and its co-secreted peptides oxyntomodulin and glicentin (15–17), together with an insulinotropic resistance to GIP in the pancreas (18) which lead to a deficient incretin system, purportedly causing the disease (19, 20). Despite being still largely unknown how hyper caloric diets are disrupting the incretin signaling, some authors have shown that even circadian rhythms disruption, and the saturated fat palmitate, are significant stressors capable to hamper GLP-1 secretion (21, 22).

Obesity and Type 2 diabetes are chronic diseases for which the most effective treatment is bariatric surgery. These invasive gut surgical procedures, aimed to reduce absorptive surface area of the proximal GI tract, such as Roux-en-Y gastric by-pass (RYGB) or Sleeve Gastrectomy (SLG), are associated with an improved glycaemic control, weight loss, and often with complete remission from T2DM (23).

Despite this, the complete remittance of a great fraction of RYGB patients represents a fascinating new case-series that points at the importance of the EECs and its modulation of the whole-body metabolism (24). As such, the study of this complex endocrine organ, might help us to create new pharmacological tools to amend the specific molecular axis that drive T2DM and the associated co-morbidities known to affect the cardiovascular (25, 26) and renal system (27, 28).

A panoply of contradictory studies have attempted to establish what is the possible role of GLP-1 or other gut peptides in the rapid, and long-lasting remittance from T2D after bariatric surgery, but no consensus about the identity of the molecular players has yet been reached (29–39).

Since 2005, there are on the market only two classes of drugs that attempt to bolster glucagon-like peptide-1 signaling, GLP-1 receptor agonists and DPP-IV inhibitors, for a supra-physiological GLP-1 activity. Unexpected safety-issues and important side-effects (40) prove that the peripheral hijack of this peptide is not sufficient, and does not replicate the remittance seen in bariatric surgery.

This review summarizes the most recent studies that reframe our understanding of the physiology of GLP-1 in health and disease.

CHEMOSENSATION IN GLP-1-PRODUCING CELLS

Intestinal proglucagon expressing cells were historically named L-cells more than 4 decades ago because of their large 500 nm secretory granules seen under electron microscopy (41). Today, we know that these are nutrient-responsive enteroendocrine cells that secrete a variety of peptide hormones, primarily derived from the proglucagon gene (*GCG*) (42). Once translated, the 180 amino acid long GCG protein is processed by two proteases, Psck1 and Psck3, to give GLP-1, GLP-2 but also the less studied and understood glicentin and oxyntomodulin (43). Other peptide hormones, such as insulin-like peptide 5 (INSL5) (44, 45), PYY (46), GIP and neurotensin (17, 47) can be co-expressed with the *GCG* products depending on the topographical localization of the cell; surprisingly, it appears that GLP-1 and PYY can be excreted independently possibly due to the existence of compartmentalized secretory vesicles (48).

There appear to be considerable species-specificity in terms of anatomical localization of GLP-1 production as summarized in **Figure 1**. Independently of other hormones, in mice the distal colon and rectum show the higher levels of GLP-1 per gram of tissue. Conversely, in rats the distal ileum and in pigs the caecum are the anatomical regions with the highest amounts of GLP-1 (49). In humans, the density of GLP-1 and PYY positive cells increase steadily along the small intestine, decreasing in the colon, and then raising again reaching a maximum density in the rectum with the highest values of around 150 GLP-1-expressing cells per square millimeter. Curiously in type 2 diabetes, an equally distributed gradient of GCG and PC1/3 mRNA appears upregulated, but with normal GLP-1^+ cell densities, indicating a possible translational resistance (51).

The L-cells derived cocktail of hormones is believed to play pivotal roles in digestion, for example slowing down the GI motility (PYY) and suppressing the appetite *in vivo* (GLP-1, oxyntomodulin, PYY), apparently in response to direct sensing of the gut luminal content via G-protein coupled receptors or through neuronal circuits (43, 52).

Current *in vitro* technologies are not capable to support for long-term *ex vivo* the growth of isolated GLP-1 producing-cells. The available knowledge about the biology of GLP-1 is primarily drawn upon studies operated with the murine-derived GLUTag or STC-1, and the human-derived NCI-H716 cell lines. It is important to understand that these *in vitro* models express a different hormonal cocktail and respond to different chemical stimuli than intestinal L-cells *in vivo* (53, 54). Primary cultures are another useful short-term system; nonetheless GLP-1-producing cells amount to only 1–2% of the whole cultured mucosal population, with considerable intra and inter-assay variability (53).

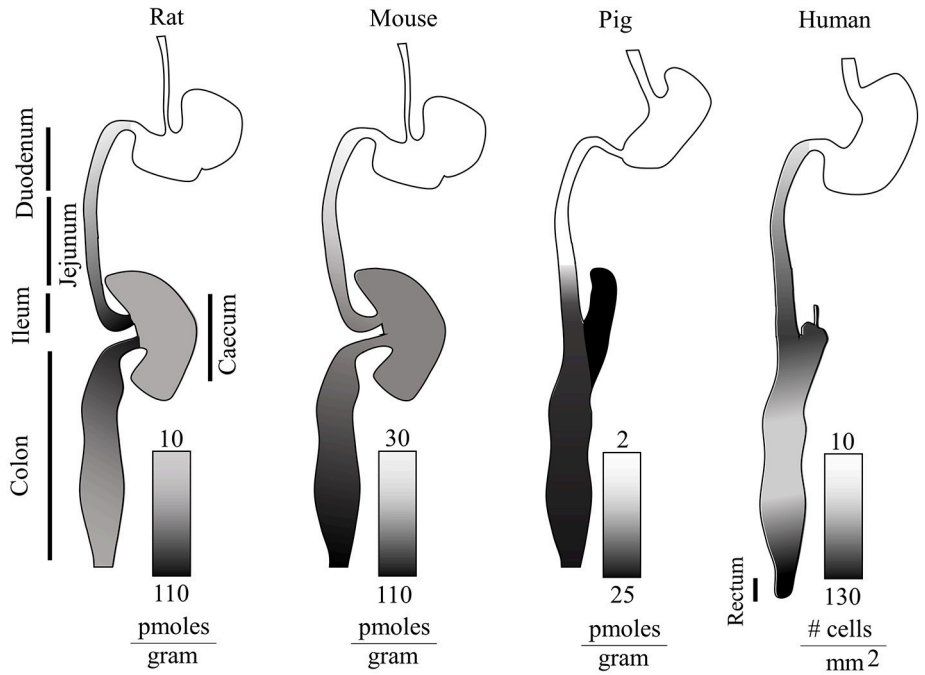

FIGURE 1 | Intestinal glucagon-like peptide-1 expression across species. Total GLP-1 expression along the rat, mouse, pig and human intestinal tracts (relative lengths not to scale) is displayed with gradients as individually indicated in figure. The rat GI tract shows the highest levels of GLP-1 in the ileum and proximal colon. On the other hand the murine gut, displays the highest GLP-1 levels in the distal colon. The porcine intestine shows highest levels in the caecum and distal colon, and virtually none in the proximal small intestine. In humans, a steady increasing gradient along the small intestine is followed by a decrease in expression in the colon, and a second steeper gradient culminating in the rectum with the highest GLP-1 expression (49–51).

The more physiologically relevant studies make use of *in vivo* transgenic mice, *ex vivo* perfused intestines or, more recently, crypt organoids derived from human, mouse or porcine guts (55).

In situ immunostaining and FACS studies have demonstrated that the hormonal secretome of GLP-1-secreting-cells is anatomically dependent. In the upper gut where these cells are more sparse and rare, GLP-1 is co-expressed with GIP, a K-cell feature, but also with cholecystokinin (CCK) and Neurotensin (NT). Conversely in the colonic mucosa, GLP-1 co-localizes with PYY, CCK and the orexigenic Insulin-Like peptide 5 (INSL5) (4, 43, 45, 53, 56, 57). Interestingly, colonic L-cells possess twice as much total GLP-1 compared to L-cells from the upper GI tract (53). Furthermore, considering the differential response to glucose, it is clear that the physiology of this population of EECs is distinct, and evolved under a different evolutionary pressure dictated by the exposure to a different luminal content (53, 58).

L-cells are known to modulate the release of their hormonal cargo in response to the activation of a plethora of receptors capable to sense fats, carbohydrates, proteins and many other compounds. Enteroendocrine cells, like other endocrine, muscle and neuronal cells, are electrically excitable. Membrane depolarization, triggered by a ligand-bound receptor, results in a spike of intracellular calcium (Ca^{2+}) which leads to the fusion of the endocrine granules with the lateral and the broader basal side, resulting in the discharge of a hormonal cargo in the capillaries of the mucosa.

Surprisingly, the EECs in the colon have been demonstrated to physically connect through a basal process named Neuropod, with afferent nerve cells residing in the lamina propria, defining a neuroepithelial circuit that expands the physiology of these cells (59). In fact, the idea of a direct neuronal regulation has been demonstrated decades ago in rats, where a bilateral vagotomy massively downregulates circulating PYY and GLP-1 levels after a glucose load (60). Furthermore, intracerebral acute, but not chronic administration of GLP-1 in mice, improves pancreatic glucose stimulated insulin secretion (61).

GPCRs AS MOLECULAR TASTANTS

G-protein coupled receptors (GPCRs) are evolutionary ancient proteins spanning seven times across the plasma membrane of virtually any known cell type. In metazoans, these proteins evolved into thousands different molecular transducers capable to translate the presence of extracellular molecules into intracellular cascades of messages amplified by different G-proteins, which in turn enforce a myriad of different cellular processes via secondary messengers (62). The transmembrane domain of these chemosensors being exposed to a tighter evolutionary pressure lead to a relative evolutionary stability of the same 3-dimensional structure. On the contrary, the extracellular facing portion is what primarily defines the identity of a myriad of different receptors, capable to sense a panoply

of molecular entities ranging in size from a single atom to hundreds aminoacids long proteins. The intracellular portion of these nano-sensors, has evolved in humans in a complex hub that triggers multiple molecular cascades that results in short-term and long-term modifications of the target cell and even the whole-body metabolism.

Different receptors, expressed by the same cell type or tissue, can trigger the same molecular cascade. With this notion, the study of these molecular transducers has been approached by some authors in recent years from a top-down point of view, whereby sub-type specific, allosteric positive or negative modulators (PAM, NAMs), as well as direct agonists, are utilized as tools for pathway dissection and analysis (63, 64). In the last decade, technological advancements in techniques such as circular dichroism (65), Cryo-electron microscopy (Cryo-EM) (66) and crystallography (67) have expanded our understanding of the physiology of multiple chemosensors expressed by L-cells, which led to the discovery of new molecular tools with possible future clinical applications in diseases such as type 2 diabetes (64, 68–70).

The expression of different GPCRs to restricted anatomical regions, such as the enteroendocrine cell system, is a finely tuned system that evolved in metazoan. Macronutrients, bile acids (BAs), and microbiota-derived compounds activate many of these GPCRs expressed by GLP-1 expressing cells (71). Nonetheless, not all intestinal stimuli signals through these chemosensors; for example glucose induces the release of GLP-1 from human duodenum and ileum via electrogenic transporters (SGLT1) and voltage-gated Calcium and Sodium channels responsible for the membrane depolarization and hormonal release (53, 72).

The main G protein-coupled receptors which activation appears to cause the release of GLP-1 are: GPRC6A (73), GPR40-41-42-43-93-119-120 (43), GPR142, GHS-R1A (74), Tas1R2-Tas2R3(T1R2-T1R3) (75), GPBAR1 (TGR5), and CasR (6, 76, 77) (**Table 1**). The functional differences seen between Jejunum-Ileal and colonic GLP-1 producing cells, could be explained by a different pool of GPCRs, or possibly by the presence of heteromers displaying a more complex pharmacology than with each individual receptor.

A summary of the recognized main activities of all the major GLP-1-secreting receptors, including the GIPR (93, 94), is shown in **Table 1**.

Many of these chemosensors are also expressed by other enteroendocrine cells, so that the same dietary ligand traveling along the GI tract, leads to the release of multiple hormones.

There are some receptors, such as GPRC6A, with a pleiotropic distribution and still a limited understanding of its physiology. GPRC6A is highly expressed in GLUTag cells, and its activation by L-ornithine has shown to induce GLP-1 secretion (102). Nonetheless, mice deficient for the receptor, show no difference in responsiveness to both L-ornithine and L-arginine (103).

THE PHYSIOLOGY OF GLP-1

In the last three decades a major tenet seeing GLP1 $(7\text{-}36)_{NH2}$, GLP1 (7-37) and the Gastric Insulinotropic Peptide (GIP) as the major contributors of the physiological incretin effect has reached widespread consensus (104). The remaining Glucose-stimulated insulin secretion (GSIS) appears to be enhanced by nutrients, hormones such as CCK, bile acids and endogenous ethanolamides. Animal models show compensatory mechanisms by which, in absence of a major incretin axis, other minor pathways are promoted in the β-cells to maintain their metabolic activity; namely proteins such as GPR119, or the CCK A receptor itself are upregulated, implying a highly plastic metabolic adaptation (105).

Multiple cell types found in the enteroendocrine cell system, the pancreatic islets or the brain have been shown to express the GCG product, a 180 aminoacids long peptide known as proglucagon (PG) (106, 107), which gets trimmed tissue-dependently into at least 6 different bio-active peptides, namely glicentin, oxyntomodulin, glucagon, miniglucagon, GLP-1 and GLP-2 (108, 109). The post-translational processing of the preproglucagon gene into the individual peptides is controlled by two distinct serine proteases, specifically prohormone convertases named Psck1/3 and Psck2, also known as PC1/3, or just PC1, and PC2 respectively (107, 108, 110). PC1/3 and PC2 are responsible for the metabolism of a plethora of peptide pro-hormones, including insulin and GCG among others (111). In particular PC1/3 expressing cells, such as intestinal L-cells and pancreatic β-cells, produce GLP-1, GLP-2 oxyntomodulin and glicentin (110, 112), while PC2 action on PG results in the production of glucagon and its active metabolite mini-glucagon (113, 114). Differential expression of PC genes regulates the hormonal output, and indeed it has been proven that both are expressed along the intestine, with PC1/3 positive cells found more distally than PC2 expressing cells (51), likely secreting glucagon (115). Indeed, the RYGB surgery removes the biggest pool of PC2/glucagon expressing cells from the exposure to nutrients, possibly contributing to the surgical success.

Active GLP-1(7-37), in human and mice is largely metabolized by the enzyme peptidyl-glycine α-amidating monooxygenase (PAM) into the equally active GLP-$1(7\text{-}36)_{NH2}$ (49, 116). Both these peptide species are trimmed at their N-term, and inactivated by the ubiquitous protease dipeptidyl-peptidase-IV (DPP-IV), found in the intestinal capillaries, vena porta and liver. Indeed, it has been estimated that just 10-15% of the secreted GLP-$1(7\text{-}36)_{NH2}$ reaches the systemic circulation (117), with some authors reporting meager peripheral meal-induced changes in both healthy and diabetic people (118). Furthermore, the DPP-IV product, GLP-$1(9\text{-}36)_{NH2,}$ is trimmed into GLP-$1(28\text{-}36)_{NH2}$ and GLP-$1(32\text{-}36)_{NH2}$ by another ubiquitous protease, known as NEP24.11, CD10 or also Neprilysin among other names (119, 120).

Indeed, these once thought inactive metabolites of the recognized GLP-1 receptor agonist GLP-$1(7\text{-}36)_{NH2}$ have recently shown to possess multiple beneficial properties. The 9 aminoacids long GLP-1(28-36) protects β-cells from glucolipotoxicity (121), diet-induced steatosis of the liver (122), improves hepatic glucose tolerance in diabetic mice (122–124). Similarly, the 5 aminoacids long GLP-$1(32\text{-}36)_{NH2}$ improves glucose disposal, increases energy expenditure and protects β-cells in a diabetic environment *in vivo* (125–127). Indeed GLP-1(9-36) pharmacodynamics studies in human might be partially explained by the activity of its metabolites (128).

TABLE 1 | Demonstrated primary effects of the major GLP-1-stimulating receptors.

Receptor	Ligand	Effect	Experimental condition	References
FFAR1/GPR40	Palmitate	Insulin ↑, glucagon ↑, somatostatin↑	*Ex-vivo* human islets	(78)
	Free fatty acids	GLP-1 ↑, GIP ↑	*In-vivo* mouse	(79)
	Long chain fatty acids	CCK ↑	*Ex-vivo* murine duodenal I cells	(80)
FFAR2/GPR43	Inulin	PYY ↑	*In-vivo* diabetic mouse	(81)
	Propionate	PYY ↑, GLP-1 ↑	*Ex-vivo* murine colonic Primary cultures, & *in-vivo* murine and rat	(82)
FFAR3/GPR41	Propionate	PYY ↑, GLP-1 ↑	*Ex-vivo* murine colonic primary cultures	(83)
FFAR4/GPR120	α-Linolenic acid	GLP-1 ↑	*In-vivo* mouse	(84)
	Lard oil, corn oil	GIP ↑, CCK ↑	*In-vivo* mouse	(85, 86)
GPR119	Oleoyl-LPI, OEA	GLP-1 ↑	*In-vitro* murine GLUTag, *ex-vivo* human colon	(87, 88)
	AR231453, AR435707, AR440006, OEA, 2-0G	PYY ↑, GLP-1 ↑, GI motility ↓	*Ex-vivo* murine gut, *in-vivo* healthy and diabetic mouse, *ex-vivo* human colon	(89, 90)
	Hypergl* + AR231453	Insulin ↑	*In-vitro* murine MIN6	(88)
	Hypergl. * Compounds A/B$^{\Delta}$	Insulin ↑	*Ex-vivo* rat pancreas	(91)
	Hypogl.** Compounds A/B$^{\Delta}$	Glucagon ↑	*Ex-vivo* rat pancreas	(91)
	DS-8500a	Insulin ↑, glucagon ↑, GLP-1 ↑, GIP ↑, PYY ↓	Type 2 diabetic humans	(92)
GIPR	Hypogl.** + GIP	Glucagon ↑	Type 1 diabetic humans	(93)
	Hypergl.* + GIP	Insulin ↑, somatostatin↑	Healthy humans	(94)
	GIP	IL-6 ↑	*Ex-vivo* human, and murine α-cells	(95)
GLP-IR	GLP-1	Insulin ↑, somatostatin↑, glucagon ↓	*Ex-vivo* healthy murine pancreas	(96)
	GLP-1	Appetite ↓	*In-vivo* intracerebral rat	(97)
	GLP-1	GLP-1 ↑	*In-vitro* murine α-TC 1-6	(98)
	Exendin-4	Glucagon ↓	*Ex-vivo* healthy rat pancreas	(99)
	Exendin-4	Glucagon ↑	*Ex-vivo* diabetic rat pancreas	(99)
TGR5	Hypergl.* + INT-777†, or LCA§	GLP-1 ↑, insulin ↑	*Ex-vivo* healthy human, and murine diabetic islets	(100)
	Taurodeoxycholate	GLP-1 ↑	*Ex-vivo* murine primary ileal cultures	(101)

*Analytes are indicated as up (↑) or down (↓) regulated. All in-vivo, or in-human studies, indicate peripheral plasmatic levels. *(Hypergl.) and **(Hypogl.) indicate conditional presence/hyperglycaemia, or absence of glucose/hypoglycaemia. §(LCA) lithocolic acid, †(INT-777) semisynthetic bile acid, (GSIS) Glucose-stimulated insulin secretion. Δ(Compounds A and B) are experimental GPR119 agonists described by Li et al. (91).*

These metabolites have possibly important implications for any future treatment of metabolic pathologies such as type 2 diabetes, where our understanding of the pharmacokinetic and pharmacodynamics in humans is virtually absent (128).

In healthy humans, intact GLP-1(7-36) $_{NH2}$ is mainly released by intestinal EECs after the ingestion of food, especially meals rich in fat and proteins (14, 129). Other stimuli, such as physical activity, are also capable to raise its plasmatic levels for up to 90 min after exercise (130).

This hormone generates both short-term and long-term pleiotropic effects. GLP-1 stimulates the β-cells to produce Insulin, blocks pancreatic α-cells' glucagon release via somatostatin (96), slows down gastric emptying (131), improves peripheral glucose tolerance (132), suppresses appetite in the hypothalamus and amygdala (97), increases β-cell mass, GSIS, and elicits protection from glucolipotoxicity (133) and apoptosis (134). Curiously, it also regulates bone physiology (135), and shows anti-inflammatory properties (136).

On the other hand, the most abundant DPP-IV-processed metabolite GLP-1 $(9\text{-}36)_{NH2}$, has also been reported to have biological activities, protecting human aortic endothelial cells and cardiomyocytes *in vivo* in dogs (137) and *ex vivo* in mice (138) and rats (139), even in the absence of a GLP-1 Receptor (139, 140). Some authors postulate the existance of an unknown GLP-1$(9\text{-}36)_{NH2}$ receptor (141, 142), because indeed this cleaved peptide is found in peripheral blood at one order of magnitude higher concentrations than "active" GLP-1 $(7\text{-}36)_{NH2}$ and shows cardioprotection, antioxidant properties (138) and appears capable to also inhibit hepatic neoglucogenesis (141).

GLP-1 $(7\text{-}36)_{NH2}$ itself is known to have general protective and modulating cardiovascular effects (143), as shown by different commercial GLP-1 mimics with proven cardioprotection type 2 diabetes (144).

In healthy fasted individuals, it is recognized that peripheral plasmatic active GLP-1 $(7\text{-}36)_{NH2}$ plasmatic levels hover around 5 pM, but within 5–10 min after an oral glucose load, they start to rise, up to a maximum of less than 10 pM after 40–90 min, and slowly descend back to baseline values in 150 min. On the other hand, the cleaved GLP-1 $(9\text{-}36)_{NH2}$ summed to the GLP-1 $(7\text{-}36)_{NH2}$ to give what is normally referred to as total GLP-1 levels, raise up to more than 40–60 pM (108). In perspective, GIP and Insulin show much broader dynamic ranges, with post meal levels reaching 300 and 400 pM respectively, from their baselines <20 pM within 30 min post glucose ingestion (108, 145). Curiously, some bariatric RYGB patients experience up to a 10-fold increase in post-meal active GLP-1 plasmatic levels (from fasting 5 pM to post-prandial 30–65 pM) (146), and have a 2- to 3-fold higher glucose-stimulated Insulin secretion (147), which in some diabetic patients results in GLP-1-mediated hyperinsulinemic hypoglycaemia that requires GLP-1 antagonism or surgical reversal of the intestinal anatomy (148).

Different authors consider the success of surgical intervention a consequence of a major change in gut hormonal profile, primarily a supra physiological post-prandial GLP-1 secretion (29, 30). This reasoning fits with the observation that type 2 diabetic patients display a shorter post-prandial peak of GLP-1, hence they are deficient for the longer response seen in healthy individuals. Multiple groups describe diabetic patients with lower plasmatic GLP-1 but heightened GIP levels and β-cell resistance to the stimulatory effect of both GLP-1 and GIP (18, 149–153).

Nonetheless, different animal models deficient for GLP-1 signaling, in addition to human studies, prove the dispensability of GLP-1 for surgical success (31–34), questioning the causative nature of GLP-1 for the reported metabolic benefits.

On the other hand, PYY has been proven to be upregulated, and necessary, for RYGB mediated restoration of the diabetic islets, and overall cure of diabetes in rats (35) and humans (154).

Another important source of endogenous GLP-1 is the brain, a tissue where it acts as a neurotransmitter. Indeed central GLP-1 production appears essential, since peripheral GLP-1 is assumed to not be able to cross the blood-brain barrier (BBB). In particular, neurons of the hindbrain found in the nucleus-tractus solitarius (NTS) secrete GLP-1 and activate hypothalamic neurons of the paraventricular nucleus (PVN), resulting in satiety (155, 156). Indeed it is clear that PC1/3 dominant neurons of the NTS express also other the PG peptides oxyntomodulin, glicentin, and GLP-2 together with GLP-1 (157). Although expressed at much lower levels, PC2 activity has also been recognized in these neurons, and traces amounts of glucagon might have important implications.

NTS neurons-derived GLP-1 appears to reach out to multiple locations within the central nervous system (CNS), which have been proven to express the receptor, and be activated after a central administration of GLP-1 receptor agonists. These areas include the NTS itself, the supraoptic nuclei, the arcuate nucleus (ARC) and the area postrema (AP) other than corticotropin-releasing hormone (CRH) PVN neurons (158, 159). Beyond satiety, this signaling appears to be a key factor for neuroprotection (160) insulin sensitivity and glucose metabolism (158).

Curiously, the feeling of satiety, is also achieved by another neurotransmitter, the Cocaine- and amphetamine-regulated transcript (CART) (161). This peptide, acts also as a hormone, and is expressed by both β-cells and intestinal GLP-1 and GIP producing cells causing GLP-1 secretion *in vivo* via a yet unknown GPCR (162).

It is not entirely clear to what extent endogenous GLP-1 activates all the reported GLP-1 receptor expressing neurons and to what extent it depends on the CART peptide especially in type 2 diabetes or obesity. Nonetheless, some commercial mimics of GLP-1, such as Liraglutide, even when administered peripherally, appear to cross the BBB and activate neurons within the ARC resulting in GABA dependent inhibition of neuropeptide Y (NPY) and agouti-related peptide (AgRP) secretion. This signaling has proven to be essential for the Liraglutide mediated weight loss in rats (163). GLP-1R expressing hypothalamic neurons have proven dispensable for the beneficial metabolic activity of both BBB permeable Liraglutide and Exending-4 (164).

Singularly, BBB impermeable mimics of GLP-1 have still shown to activate GLP-1 Receptor expressing neurons (165), but they require a functional gut-brain axis through the vagus nerve (166). In particular, vagal afferent neurons expressing the GLP-1R are necessary for GLP-1 mediated induction of satiety (167) but not glucose lowering effects (168).

The complex inter-organ pharmacokinetic of GLP-1, compounds into a convoluted pharmacodynamics encompassing multiple metabolic systems.

Indeed the GLP-1$(7\text{-}36)_{NH2}$ receptor, a GPCR, is found to be expressed by a wide range of tissues and cells such as: α, β, and δ-cells (169), sinoatrial node myocytes, arterial smooth muscle cells of lungs and kidneys, megakaryocytes, macrophages, monocytes, lymphocytes, gastrointestinal tract mucosa [mainly Brunner's gland in the duodenum, but also in the parietal cells of the stomach, jejunum ileum and the nerve plexus around the small and large intestine (170, 171)], central nervous system [neocortex, cerebellum, thalamus, amygdala, area postrema, hypothalamus, hippocampus, nucleus tractus solitarius (158)], peripheral nervous system (myenteric plexus) and in the skin (14, 172–176).

Counterintuitively, mice completely defective for the GLP-1 receptor were reported to be protected from high-fat diet-induced peripheral Insulin resistance (177) and, consistently with this, central inhibition of GLP-1R signaling with the antagonist exendin 9-39 improves glucose tolerance and glycaemia (178). Conversely, mice defective for both the receptors for glucagon and GLP-1, or GLP-1 and GIP, show a highly plastic entero-pancreatic system that adapts and gives these animals no overt phenotype in terms of glucose homeostasis (105).

Nonetheless, the pharmacological activation of the GLP-1R is clinically beneficial (179), offering an improved glycaemic control with lower cardiovascular morbidity and without the risk of hypoglycaemia associated with some current anti diabetic drugs (173). Furthermore, being an appetite suppressant, GLP-1 signaling also helps to lose body weight, especially if in combination with metformin. Conversely, anti-diabetic drugs such as sulfonylureas, or Insulin, are known to induce not only weight gain (180, 181), but also an increased risk of

hypoglycaemic events (182). Pharmacological activation of the GLP-1 Receptor has also shown to help exogenous insulin in the control of glycaemia in patients with type 1 diabetes, by slowing the gastric emptying and blocking glucagon secretion (183, 184).

Currently, six different peptide GLP1-Receptor agonists are on the market, with more in clinical trials. In particular, two short-acting formulations of Lixisenatide and Exenatide and four long acting preparations of Exenatide, Liraglutide, Dulaglutide and the most recent and successful Semaglutide, were approved in October, 2017 for the North American markets by FDA[1] (25, 185). The first GLP-1 analog to be approved by FDA in 2005 for the management of Type 2 diabetes was the chemically synthesized Exenatide under the name of Byetta (186), a formulation of the DPP-IV resistant peptide discovered in the gila monster *Heloderma suspectum* saliva in 1992 (187). Despite the longer half-life in serum, Byetta needs to be injected twice a day. In the last decade, formulations with extended release entered the market with once-weekly self-administrations pens.

Pleiotropic beneficial effects have been reported for this class of drugs. Beyond the improved glycaemia control, essential for the short term treatment of diabetes (188), different GLP-1RAs are powerful clinical tools for the management of diabetic kidney disease (DKD) (28, 189) non-alcoholic steatohepatitis (NASH) (190), neuroinflammation (191), obesity and cardiovascular disease (192–195).

Although GLP-1RA are improving the lives of patients affected by type 2 diabetes or the metabolic syndrome (196), the physiology of GLP-1 is far from being clear.

More recent data suggest how the unimolecular co-activation of GLP-1 and GIP receptors, has powerful anti-diabetic effects superior to either agonism (197). Furthermore, oxyntomodulin is a natural dual-agonist of GLP-1 and glucagon receptors and displays anti-diabetic properties in humans (198, 199). Upon this finding, a tri-agonist peptide, targeting the receptors of GLP-1, GIP, and glucagon was created (200). The *in vivo* effects of this drug are unparalleled, even superior to what can be achieved with the dual agonists for either combination. The synergistic activation of these three important receptors is capable to revert diet-induced obesity, cognitive impairment and T2D in mice models, warranting future human studies (201, 202).

EXPANDING THE PHYSIOLOGY OF GLP-1

When examining the physiology of glucagon-like peptide-1, it is important to consider that there is an expanding body of evidence that questions its systemic endocrine physiology (203, 204). Pancreatic α-cells have been demonstrated to express and secrete not only GLP-1 (205, 206), but also PYY (35) GIP (207, 208) mini-glucagon (209) or even Xenin (210) together with glucagon (**Figure 2**). The key protease responsible for the processing of the proglucagon peptide into GLP-1 is Psck1/3, which has shown to be upregulated in α-cells during hyperglycaemic, hyperlipidemic, or inflammatory conditions to promote glucose-induced glucagon suppression, a compensatory response to a metabolic insult as in type 2 diabetes (205). Insulin itself has shown to modulate PC1/3 expression to possibly aid its own metabolic activity (211).

Recently, the whole dogma of the role of intestinal GLP-1, envisioning the traveling from the gut to the liver and ultimately reaching the pancreatic β-cells to bind its GLP-1R has been questioned in transgenic mice (204). Indeed, since both DPP-IV degrades and NEP24.11 degrade GLP-1 within seconds, the possibilities of any intestinal GLP-1 to reach the system circulation and then the islet microcirculation are doubted. Besides, it is important to consider that intestinal GLP-1 has a local concentration in the nM range (10–100 pico moles per gram of tissue, see **Figure 1**), further advocating that the main action of this protein have evolved to be locally restricted.

Animals deficient for the GCG gene in the intestine, still experience a normal incretin effect disrupted with the GLP-1R antagonist Exendin (9-39) (204). This indicates that it is the intra islet, α-cell derived GLP-1 that shows the meal-induced insulinotropic properties. A critic to the use of a murine model deficient for intestinal GCG products, would be that other gut hormones might compensate for the lack of a functional GCG gene in that tissue, hence explaining the normalized incretin effect. Indeed other gut hormones such as GIP must be responsible for the incretin effect to a higher degree than once thought. Nonetheless, it is also clear that intra-islet GLP-1R signaling is essential for GSIS, with more evidence that an intra-islet paracrine GLP-1 signaling is physiologically present (212, 213) and necessary for β-cell health under metabolic (214).

In contrast, mice deficient for GLP-1R only in β-cells have a normal incretin response and oral glucose tolerance, indicating the dispensability of intra-islet signaling of GLP-1 for the incretin effect. Interestingly, these same animals have an improvement of their glucose tolerance in response to oral DPP-IV treatment, but not to subcutaneous GLP-1 mimics, indicating how the former relies completely on localized, non β-cell GLP-1R (215).

There are still multiple gaps into our understanding of how different GLP-1 producing tissues communicate, especially in the brain to islet axis. It is known that acute, but not chronic, central GLP-1 receptor activation directly modulates glucose-induced Insulin secretion implicating a direct brain to islet neuronal communication (61).

On the other hand, chronic GLP-1 activity in α-cells increases its own secretion, feeding an autocrine loop that gets overstimulated with the use of exogenous synthetic GLP-1R agonists [(98); **Figure 2**]. Curiously in diabetic rats, it has recently been shown that this loop might indeed induce the production of more glucagon than in healthy animals (99).

It has been known for more than two decades and has been confirmed more recently, that an infusion of GLP-1(7-36)$_{NH2}$ has insulinotropic and glucagonostatic effects. This is seen when the plasmatic levels are above 50–60 pM, equivalent to more than five times the levels seen post-prandial in healthy individuals challenged with a bolus of glucose, or 10-fold their basal levels (153, 216), adding further doubt to the physiological hormonal dogma of intestinal GLP-1. Considering the mounting

[1]http://press.novonordisk-us.com/2017-12-5-Novo-Nordisk-Receives-FDA-Approval-of-OZEMPIC-R-semaglutide-Injection-For-the-Treatment-of-Adults-with-Type-2-Diabetes

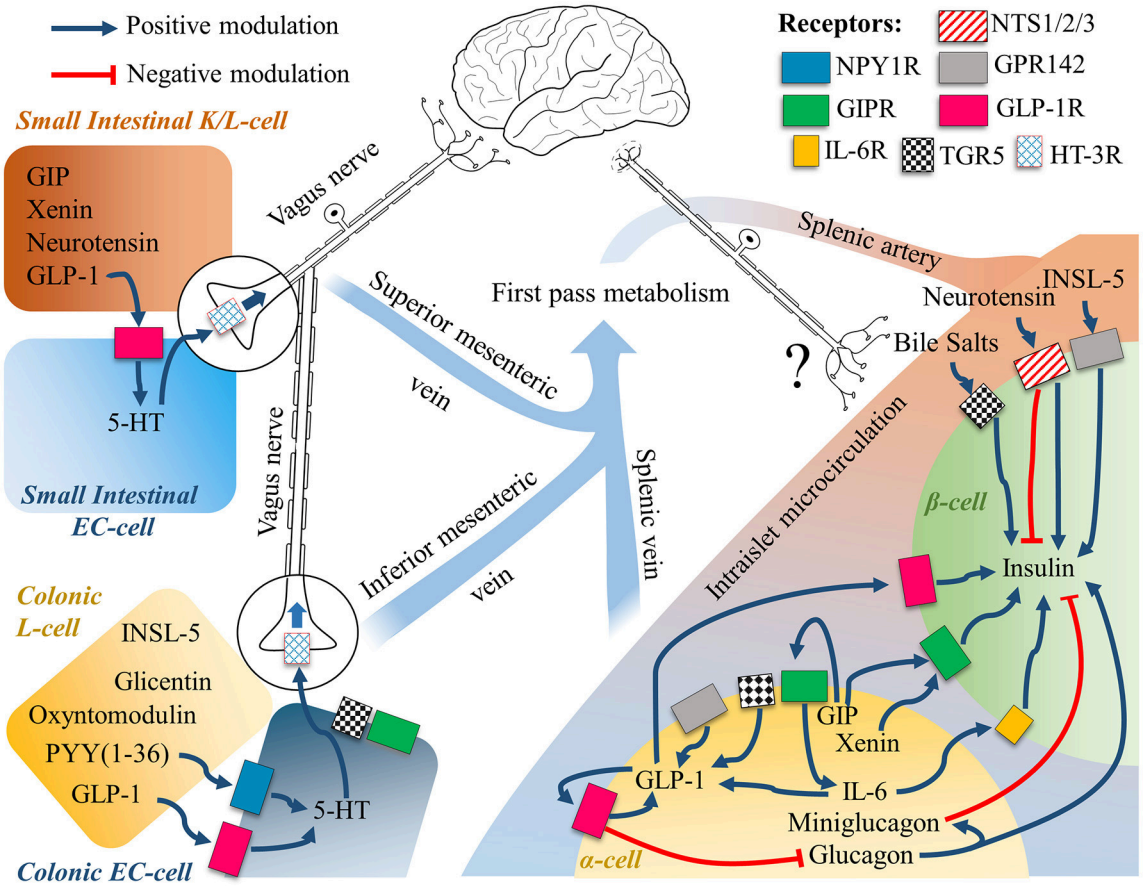

FIGURE 2 | The gut-brain-islet axes of GLP-1. The intestinal EECs secretome is subject to first pass metabolism, while intraislet signaling relies on paracrine signaling. Intestinal cells are known to communicate with the Enteric Nervous System, and the Central Nervous System through the Vagus Nerve. Neuronal engagement between the gut lumen and the islets of Langerhans is a possible compounding explanation to the incretin effect, whereby the mechanistic of the single molecular players are still largely unknown. See text for further details.

evidence, it is clear that we need to understand what hormonal and/or neuronal signals are bridging the gut luminal content to the insulin secretion explaining the incretin effect. Given that Intestinal oxyntomodulin, glicentin, glucagon and GLP-1 expression have proven to be dispensable in mice (204); other intestinal hormones such as GIP, PYY, Neurotensin, INSL-5 or the GIP co-secreted Xenin (217) might play an important role (**Figure 2**). Currently, not much is known about the physiology of Neurotensin, INSL-5 and Xenin. The first two have been reported to be co-expressed with GLP-1 in the small and large intestine respectively, with Neurotensin being reported also in pancreatic β-cells (210), while Xenin in a sub population of duodenal GIP positive cells and α-cells. Neurotensin levels are correlated with leptin (218), rise in response to fatty meals, signals through two different G-protein coupled receptors known as NTSR1 and 2, and a third single transmembrane receptor, NTSR3, also known as sortilin (219). All of these receptors are expressed by pancreatic β cells, where their activation appears to mediate insulin release at low glucose levels and blockage at high levels (219, 220), (see right side of **Figure 2**). On the other hand, INSL-5 targets a GPCR known as GPR142, also known as RXFP4, a receptor found to be expressed by the NCI-H716 cell line (54), and both α and β-cells in the pancreas, and its activation directly stimulates the expression of GLP-1 and insulin, representing a possible new pharmacological tool for the treatment of type 2 diabetes (77, 221), and supporting a possible role for INSL-5 in the incretin effect. Xenin is another gut-derived food-induced peptide known to potentiate GIP activity (222, 223). Considering that α and β-cells express GIPR (224) and that the GIP-potentiating activity of Xenin has been reported to be lost in human diabetics (223), it appears to be a critical player in this disease, likely involving the activity of GLP-1.

In addition, both *in vitro* and *in vivo* Interleukin-6 (IL-6) has shown to be a powerful GLP-1 secretagogue, capable to positively modulate both the proglucagon gene, and the expression of PC1/3 in α-cells and intestinal L-cells (225, 226). Indeed, GIP has shown to not only be co-expressed with GLP-1 and glucagon in α-cells (207); it also stimulates in an autocrine/paracrine fashion the expression of IL-6 in the same α-cells, thus indirectly acting as a GLP-1 secretagogue (95).

IL-6 has shown also to induce the secretion of intestinal GLP-1, indirectly via the release of adipocytes derived Leptin (227).

Curiously, it was recently reported that this pro-inflammatory cytokine, IL-6, similarly, but independently from GLP-1, slows gastric emptying (228). Furthermore an inflammatory status, as seen in pathologies such as type 2 diabetes, might compromise the gut mucosal permeability, leading to the exposure of intestinal EECs to luminal LPS, and a TLR4-mediated release of GLP-1 (229). This is consistent with the knowledge that GLP-1, as well as glucagon, has shown to possess powerful anti-inflammatory properties *in vivo*, an area that hold with vast therapeutical potential (136, 230).

Ghrelin is another possible player, since it has been proven to be expressed not only in the gut, but also in a distinct subpopulation of islet cells named ε-cells (231) and, being known to be a stress-induced (232) GLP-1 secretagogue (233, 234), it might play an important role in the intra-islet signaling.

Recently, it has been demonstrated that mice with a deletion of the GLP-1 receptor only in β-cells, are resistant to the beneficial anti-diabetic effect of a vertical sleeve-gastrectomy (36), suggesting how GLP-1 activity in β-cells is key to the bariatric surgery success. It is not known if intra-islet α-cells production of GLP-1 is affected by the surgical procedure or, more importantly, how this axis is impaired in the metabolic syndrome, type 2 diabetes and related pathologies.

It appears that only in RYGB and SG patients intestinal derived GLP-1 has a true endocrine role, while in healthy individuals, localized, paracrine and neuronal signals primarily define the GLP-1 physiology.

It is therefore clear that currently available GLP-1RAs, mimicking on the peripheral action of GLP-1 $(7\text{-}36)_{NH2}$, not only ignore the yet unknown physiology of GLP-1 $(9\text{-}36)_{NH2}$ or its metabolites, but they also fail to address the tissue specific physiology of GLP-1 $(7\text{-}36)_{NH2}$, while pushing to supra-physiological limits the endocrine GLP-1 receptor axis, likely explaining the reported side-effects and only partial success in the treatment of T2D.

In addition, it is important to notice that the ubiquitous DPP-IV protease targets not only GLP-1 but also oxyntomodulin, GIP and PYY among other proteins (235). Specifically, the GLP-1 co-secreted cousin PYY(1-36), agonist of the vasoconstrictive Y(1) receptor, is physiologically trimmed by DPP-IV to give rise to the appetite-suppressant, anti-diabetic and blood-brain barrier permeable PYY(3-36) agonist of Y(2) receptor (220). It is therefore clear that pharmacological DPP-IV blockage disrupts this axis and induces hypertension (236).

Recent studies provide new evidence supporting the paracrine nature of intestinal GLP-1, whereby Serotonin-(5-HT)-secreting enterochromaffin (EC) cells are directly stimulated by locally produced GLP-1, which in turn stimulate afferent Vagal nerves (**Figure 2**) bridging the gut to brain axis. Accumulating evidence suggest that, especially in the colon, EC cells express multiple receptors for the microbiome metabolites, representing a new important link bridging the microbiome to the brain (237, 238).

A better way to amend the pathophysiology of GLP-1 reported in diabetes or other diseases, would be to induce tissue specific *de novo* GLP-1 production, leading to a more physiological and likely safer, short and medium distance signaling. Numerous attempts have been made with multiple GLP-1 secretagogues such as GPR119 agonists (239) but so far no compound has reached the market because of bioavailability issues and systemic off-target toxicity. One possible way to minimize the side-effects of the single drugs is to combine them to achieve synergistic effects, as reported recently with a combination of a DPP-IV inhibition, SSTR5 antagonism and GPR40 and TGR5 agonism, capable to raise circulatory active GLP-$1(7\text{-}36)_{NH2}$ levels to more than 300-400 pM in mice (240).

SWEETNESS IN THE GUT

Studies *in vitro* and *ex-vivo* with isolated human primary cells suggest that there are two temporally distinct pathways that lead to the glucose-stimulated release of GLP-1, similarly to what happens in β-cells with the 1st or 2nd phase insulin release. A quick mechanism independent of the cell energetical state and a slower one, metabolism dependent, mediate the release of this incretin (53, 72).

The 1st phase in the pathway of glucose signaling, sees the electrogenic sodium-coupled glucose transporters 1 (SGLT1) mediated uptake of two Na^+ ions for every internalized glucose molecule (53). This depolarization is propagated through voltage-dependent Calcium and Sodium channels, which currents lead to the discharge of the hormones containing vesicles (72).

The 2nd phase is exemplified by the absorption of simple sugars, such as Glucose or Fructose, via the facilitative transporters GLUT2 and GLUT5 respectively, which leads to an increased internal metabolism mirrored by intracellular ATP levels. This state leads to the blockage of ATP dependent potassium channels and the subsequent membrane depolarization, followed by the secretion of the hormonal cargo.

Mace et al. (241) demonstrated how diazoxide, a K^+ATP channel opener, completely abolished the glucose-dependent incretin release while a channel blocker, tolbutamide, exacerbates it in terms of secreted GLP-1, GIP and PYY.

More recent data, question the first mechanism in enteroendocrine cells. Glucose mediated GLP-1 release happens in humans only in the proximal and distal small intestine and independently of ATP mediated potassium channels closure. Furthermore, concentrations of up to 300 mM glucose do not induce GLP-1 secretion from colonic human mucosa because GLP-1 producing L-cells barely express SGLT1 (43, 53, 58, 72).

Consistently, the use of α-methyl-D-glucopyranoside (MDG), an acaloric substrate of SGLT1, within 5 min triggers the release of GLP-1 as glucose does, demonstrating how it is the sodium current that triggers the release of the incretin, and not the metabolic ATP-driven arrest of potassium currents and following calcium spike (58).

The pharmacological blockage of SGLT-1 with phloridzin, in a rat small intestine perfused system, results in just a halved secretion of GIP, GLP-1, or PYY, and the addition of phloretin,

a GLUT2 inhibitor, brings these values down to basal levels. In fact, this double blockage of SGLT1 and GLUT2, completely inhibits the responsiveness to other stimulants as well, such as sucralose, glycylsarcosine, OEA, propionate and taurocholate. The activity of the calcium channel CasR is also essential for the responsiveness to free aminoacids (241).

All these observations are challenged by longer term *in vivo* studies. Blockage of SGLT-1 markedly improves glucose-stimulated GLP-1 release if a 3-h long period is considered.

The rationale given by Oguma et al. (242) is that SGLT-1 is expressed mainly in the small intestine, hence its inactivation results in heightened luminal glucose that travels down to the colon where it someway stimulates GLP-1 release. Given the fact that SGLT-1 is barely detectable in colonic proglucagon positive cells and that potassium channels in this tissue are unresponsive to sulfonylureas, the molecular sensor(s) that causes the release of GLP-1 *in vivo,* remains elusive.

Another enigmatic G protein is α-gustducin, a key element in sweet-taste transduction pathways downstream of the heterodimer formed between the GPCRs Tas1R2 (T1R2) and Tas1R3 (T1R3).

Its expression has been reported in colonic L-cells and appears to be responsible for the glucose-stimulated release of incretins (243, 244). This is confirmed by the impaired glucose-stimulated release of GLP-1 in mice lacking either T1R3 or α-gustducin (244).

Interestingly, this axis is also activated by the disaccharide sucrose and by the non-metabolizable and therefore anergic sucralose (243). Of note also Aspartame, Acesulfame K, Glycyrrhizin and Saccharin bind the sweet receptor heterodimer Tas1R2/3 and they have shown to stimulate GLP-1 secretion in the human duodenal adenocarcinoma-derived HuTu-80 cell line (245, 246). Despite this report, other groups weren't able to replicate these results (53). Indeed, it was shown that proglucagon expressing cells, derived from the colon of Venus mice cultures, were not responding significantly to Sucralose (1 mM) in terms of both released GLP-1 and intracellular Calcium. Conversely, proglucagon negative cells responded to the sweetener. More doubts about the role of Tas1 receptors were raised after the demonstration that oral gavage with sucralose, saccharin, stevia, acesulfame potassium or tryptophan do not cause a gut incretin release in Zucker diabetic fatty rats (247).

LONG AND MIDDLE CHAIN FATTY ACID RECEPTORS

The study of the receptome of enteroendocrine cells, has provided invaluable pharmacological insight with the discovery of proteins capable to sense multiple compounds once thought to be only nutrients.

A prime example is given by two GPCRs, GPR40 and GPR120, also known as Free Fatty Acid Receptor 1 (FFAR1) and 4 (FFAR4) respectively. These chemosensors are two major molecular players in the detection of dietary, medium (C8-12) and long (C14-22) chain fatty acids (LCFA) (84, 248).

GPR40 is primarily expressed by the pancreatic β-cells, where it plays a pivotal role in FFA-mediated insulin secretion (249) but also in α-cells (78, 250), CCK (80), GIP (251), and GLP-1 (79) producing cells in the gut and in hypothalamic neurons (248, 252, 253). Animals deficient for this receptor are protected from obesity-induced hepatic steatosis, hyperinsulinemia, hypertriglyceridemia and hyperglycaemia. More than a decade ago a study showed that GPR40 mediates the long-term FFA-induced lipotoxicity seen in the diabetic islets (254); nonetheless, these findings are still under debate today. Recent data are still highly polarized, with some authors supporting (255), and others disproving this (256), or even indicating that GPR40 protects β-cells from lipotoxicity (257) rendering difficult to draw any conclusive mechanistic involvement in healthy and diabetic individuals. Nonetheless, the activation of this receptor with FFAs has demonstrated to induce the secretion of incretins (79, 258) glucagon (78, 250) and partially glucose-stimulated insulin (259, 260) reducing food intake, and lowering body weight in animals models (261). Mice without a functional GPR40 display an impaired CCK and GLP-1 secretion after an oil gavage, while surprisingly animals deficient for GPR120 display a normal corn oil-induced GLP-1 secretion (80, 262).

GPR40 is coupled to both Gq and Gs proteins and *in vivo* studies suggest how signaling through both these cascades elicits the most powerful GLP-1 secretion (258). Ligands that bind GPR40 and activate predominantly only the Gq pathway are not good GLP-1 secretagogues. Indeed recently it has been shown that dietary triglycerides appear to induce the secretion of GLP-1 via GPR40 in synergy with the Gs activating GPR119 (263). Nonetheless, chylomicrons have been reported to be powerful GPR40-Gq activators and GLP-1 secretagogues, acting from the basolateral side of the intestinal mucosa (264).

The two synthetic GPR40-specific compounds AM-1638 and AM-5262, have been found to act as double Gq and Gs agonists but also as positive allosteric modulators, capable to enhance the GLP-1-secreting capabilities of Gq-only agonists such as dietary docosahexaenoic (DHA) and α-linolenic acid (ALA), independently of the orthosteric site (265).

GPR120 shows very little sequence similarities to the other free fatty acid receptors but, likewise, is found to be expressed by the enteroendocrine cell system, especially in the colon (see **Figure 3**), but also in the lungs (267), white and brown adipose tissue (274, 275), hypothalamic microglia (253), macrophages and, contrarily to GPR40, not in β-cells but in somatostatin producing δ-cells (276). Both small intestinal GIP and colonic GLP-1 secreting cells express GPR120, and the molecular cascade triggered by this receptor has been shown to mediate dietary incretin release directly or indirectly through CCK (84–86). Interestingly, both of these two receptors are expressed only by a fraction of hormone positive EECs; in particular, it has been reported that only 3% of GLP-1 positive cells express GPR40, and 23% GPR120 (266).

GPR120 displays a ligand preference similar to GPR40; a broad range of long chain fatty acids signal through it, with some ligands eliciting more robust calcium responses than others (84). Multiple dietary compounds have shown to be powerful agonists of GPR120, such as pinolenic acid, a poly-unsaturated fatty acid

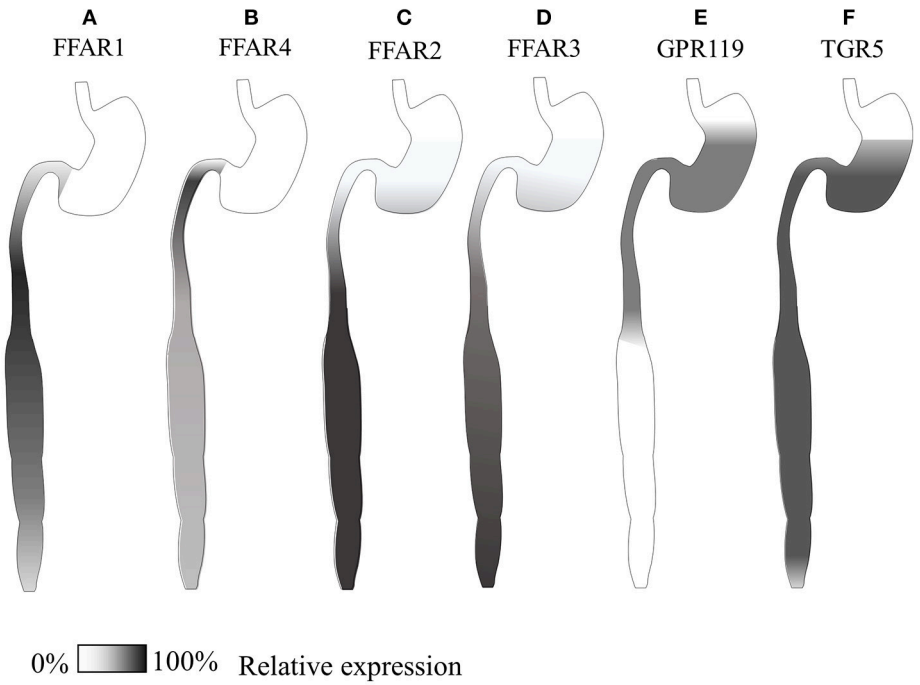

FIGURE 3 | Gastrointestinal GLP-1-secreting receptome distribution. Summary of available expression studies of different GLP-1-secreting receptors along the gastrointestinal tract. **(A)** GPR40/FFAR1 has been reported to be expressed in the small intestine in different EECs, with overall higher transcript levels than GPR120, and superior co-localization with GIP In the distal small intestine (79, 80, 266). **(B)** GPR120/FFAR4 has shown co-expression with both proximal small intestinal GIP$^+$ and large intestinal GLP-1$^+$ cells (85, 266–268) **(C)** GPR43/FFAR2 and **(D)** GPR41/FFAR3 are co-expressed by all types of enteroendocrine cells, from the stomach to the rectum, especially in the colon (83, 269, 270). **(E)** Reports of comparative GPR119 transcript are contradictory, while immunohistochemical data indicate co-localization with a minor fraction of CCK and GLP-1 positive cells mainly in the stomach and small intestine (266, 271). **(F)** TGR5, has been reported equally distributed along the whole gastrointestinal tract of dogs (101, 272, 273).

(C18:3 *trans, cis, cis* Δ 5, 9, 12) found in pine nut oil (277), or the yeast derived phytosphingosine (278).

In macrophages and adipose tissue, GPR120 mediates ω-3-mediated anti-inflammatory and insulin sensitizing effects (279, 280). Contrarily to GPR40, the genetic deficiency of GPR120 is more dramatic. Knockout animals show hyperinsulinemia and insulin resistance, hyperglycaemia and osteoarthritis (281), hepatic steatosis and therefore obesity. Furthermore, an absence of GPR120, results in an overactive glucagon signaling, explaining the hyperglycaemia (282). Indeed, in humans, a single aminoacid mutation of the receptor that hampers its signaling is associated with obesity and insulin resistance (283). Expectedly, GPR120 agonism shows powerful anti-diabetic, anorexic, and hepatoprotective properties in multiple animal models (275, 284–287), at least partially mediated by GLP-1 (288).

Considering the overlap of natural ligands of GPR40 and GPR120, it has been difficult to study them individually and understand their individual physiology, while recent data indicate that indeed these two receptors work synergistically, to exert anti-diabetic activity *in vivo* from the gut (289), and the brain (253).

Despite these advancements, in clinics there are currently no available drugs targeting GPR40 and GPR120. TAK-875, the best candidate for GPR40 which showed promising GSIS capabilities up to Clinical Phase III for the treatment of T2D, had to be halted because of hepatotoxicity and alteration of bile salts composition (290).

Despite these setbacks, encouraging animal data warrant future efforts for the development of new drugs capable to activate synergistically both GPR40 and GPR120 and mediate, through GLP-1 and other intestinal, pancreatic and cerebral peptides, better treatments for multifactorial chronic metabolic diseases.

SHORT CHAIN FATTY ACID RECEPTORS

In 1997, four 7 α-helixes transmembrane receptors, GPR 40, 41, 42, and 43 were mapped on the same locus found on the long arm of chromosome 19 (291). Soon after, different groups identified GPR 43 and 41 as the receptors for free fatty acids, which were then chronologically renamed FFAR2 and FFAR3 respectively (292–294).

Both these receptors are activated by similar types of short chain fatty acids (292), and both these signal through an inhibitory G type protein, but FFAR2 is also capable to signal

through Gq/11 proteins (293) by which it has shown to mediate GLP-1 and PYY secretion *in vitro* and *in vivo* (82, 295).

Along the gastrointestinal tract, both GPR 41 and 43 have been reported to be co-expressed, with FFAR2/GPR43 at higher levels and overall number of cells, especially intraepithelial leukocytes, while FFAR3/GPR42 is found on submucosal neurons [see **Figure 3**, (83, 295–297)]. Indeed FFAR2 holds promise for the management of Inflammatory Bowel Disease (IBD) (298) a possible side-effect of anti-diabetic treatment with DPP-IV inhibitors (299).

Feeding rats with fructo-oligosaccharide as a source of SCFAs has also shown to upregulate FFAR2 (270). Recently, both the receptors have shown to heteromerize *in vitro,* eliciting synergistic signaling and β-arrestin-2 recruitment (300). Furthermore FFAR2 activation *in vivo* with an inulin-enriched diet in mice results in PYY release and proliferation of L cells *in vitro* (81). Nonetheless, there is still some controversy on the *in vivo* involvement of FFAR2 and FFAR3 in GLP-1 modulation (301, 302), with some reports indicating that blockade of GPR43 *in vitro* releases GLP-1 (303) and others indicating different mechanisms of action, with FFAR2 releasing PYY from intestinal L-cells (81), while FFAR3 restricted to submucosal neuronal activity (295) despite its apparent expression by the majority of enteroendocrine cells (83).

In pancreatic β-cells, both GPR43 and GPR41 are expressed, and the latter antagonizes GSIS (304).

Adding complexity to the study of these receptors, there is extensive species-specificity, so that animal findings result in poorly translatable data, requiring the generation of complex human-murine chimera currently under intense study (305, 306).

Nonetheless, considering that the half-maximal effective concentration (EC_{50}) for Acetate, Propionate, and Butyrate is around 0.5 millimolar upon both GPR41 and GPR43 (292) and that the SCFA concentration in the human ileum and colon lumen is superior to 100 millimoles per kg (307–309), it is likely that both receptors are constitutively active. Obese patients, have been reported to produce more SCFAs in their intestines (310), but indeed meaningful diet-induced shifts in SCFA production fluxes have proven not sufficient to modulate peripheral levels of GLP-1 and PYY (311).

GPR42 is another G-Protein-Coupled-Receptor that was initially considered to be an inactive pseudogene derived from GPR41. In 2009, 29% of 202 human alleles of GPR42 were shown to have an inactivating single nucleotide polymorphism (SNP) at W174, and 61% with an arginine in like GPR41, resulting in a fully functional receptor, differing from it by only 5 aminoacids (312). A more recent study highlights how GPR42 is not only functional, but displays a pool of haplotypes in a great proportion of humans, with a distinct pharmacology (313).

GPR119

GPR119, also known among other names as glucose-dependent insulinotropic receptor (GDIR), was independently discovered less than two decades ago by several groups around the world and deorphanized soon after with the discovery of Oleoylethanolamide (OEA) as its first endogenous ligand (314–316).

Recently our group has demonstrated that indeed OEA is just a partial agonist of GPR119, and the biological ligand of this receptor is the lysophospholipid Oleoyl-Lysophosphatidylinositol (Oleoyl-LPI) (87). This bioactive lipid induces a powerful GPR119 mediated-GLP-1 secretion *in vitro* and *ex-vivo* from intestines of wild type, but not GPR119 deficient mice. This peculiarity is not shared by LPI species with different aliphatic chains, which have been described as the ligands of GPR55 (317).

This GPCR is primarily expressed in the pancreas by α-cells, β-cells and γ-cells (271, 318, 319), and is found at lower concentrations along the GI tract, especially in the stomach and duodenum, where counterintuitively only a minor fraction of CCK, and GLP-1 expressing duodenal enteroendocrine cells display GPR119 (266, 271). This receptor is also expressed, and hence can be studied, *in vitro,* by the human enteroendocrine cell model NCI-H716 or by the murine GLUTag cell line (320). Heterologous expression *in vitro* unveiled its constitutive activity capable to raise intracellular cAMP levels through Gαs (321) and lead to the secretion of GLP-1 and PYY (89). Rodents, contrarily to humans, express GPR119 also in some regions of the brain (316). The activation of this receptor is known to mediate glucose-stimulated insulin secretion and a glucose-independent release of incretin hormones by intestinal enteroendocrine cells (88).

Long-chain fatty acids and phospholipids like lysophosphatidylcholine (LPC), other compounds such as retinoic acid (RA) and multiple N-acylethanolamines (NAE) such as N-oleyldopamine (OLDA), palmitoylethanolamide (PEA), or oleylethanolamide (OEA), all act as endogenous ligands of GPR119. OEA is a more potent GPR119 agonist than its glycerol ester 2-Oleoyl Glycerol (2-OG) found in olive oil (322).

Indeed, oleic acid is internalized via CD36 and converted to OEA in the duodeno-jejunal enterocytes, which in turn causes satiety directly via PPAR-α (323) or indirectly through an incretin secretion mediated via GPR119 in the gut (324). Curiously, fat-induced OEA synthesis is a fairly conserved pathway in metazoan, being present in fish and extremely slow-metabolism reptiles such as pythons (325, 326).

Triglycerides, with medium length fatty acids such as 1,3 Dioctanoyl- 2 Oleoyl-glycerol, can also cause the release of GLP-1 in humans. However, this happens via the metabolized 2-OG component, since dietary medium chain fatty acid do not cause any appreciable release of incretins (322).

Counterintuitively, long term olive oil feeding does not improve glucose tolerance or insulin responses in diabetic rats (5). Indeed, more recently it has been reported that a high-fat diet enriched in oleic acid leads to an impaired endogenous OEA and other N-acylethanolamides intestinal production in mice (327), suggesting that a chronically resistance is taking place within the OEA synthesis pathway.

Surprisingly, a daily activation of GPR119 with OEA or other synthetic agonists, increases β-cell responsiveness in islets transplanted into STZ-induced diabetic mice (328).

The importance of GPR119 in the fat-induced incretin secretion is demonstrated by the impaired incretin signaling displayed by transgenic animals deficient for this protein only in PG expressing intestinal cells. Male and female mice, completely loose the GLP-1 response to an oral gavage of olive and corn oil (329).

More recently, it was reported that whole-body GPR119^{-}knockout mice are protected from high-fat induced glucose intolerance and insulin insensitivity. Interestingly, the specific ablation of GPR119 only in β-cells does not affect glucose tolerance nor insulin secretion. In fact AR231453, a selective GPR119 agonist, improves glucose tolerance and insulin sensitivity in both WT and Gpr119$^{\beta cell-/-}$, suggesting how insulin release is independent from pancreatic GPR119 but depends on gut incretin release (330).

Curiously, GPR119 activity appears to be directly dependent on the PYY receptor NPY1 (331). This phenomenon is independent of DPP-IV, the GLP-1 receptor, or the PYY related peptide NPY.

Furthermore, GPR40 also shows synergism with GPR119, mediating a more than additive GLP-1 response to triglycerides in the large intestine (263).

Agonism of GPR119 in both healthy or diabetic and obese mice, is known to improve glucose tolerance (90), or even prevent atherosclerosis in mice (332), while at the same time inducing the secretion of glucagon under low glucose levels avoiding hypoglycaemia (91); therefore since 2008, multiple agonists have been synthesized (239, 254, 333), as well as unimolecular dual DPP-4 inhibitors and GPR119 agonists (334). Despite the good results seen in rodents, species-specific pharmacology might be to blame (335).

Up to now all the prospective GPR119 agonists were plagued by low bioavailability, lack of efficacy and more importantly, cardiotoxicity which has stopped all human studies before any large scale Phase III clinical trials (239).

Despite the multiple failures, the compound DS-8500a is showing promising glucose lowering properties in Phase II clinical trials without any apparent toxicological issues in clinical trials (92).

TGR5

Bile acids (BAs) are cholesterol-derived molecules produced in the liver and temporarily stored in the gallbladder. When food is ingested, BAs are released into duodenum to solubilize dietary lipids under the form of micelles, a necessary step for the maximization of the surface-to-volume ratio of fat droplets, aiding interface-acting lipases.

Indeed the release of lipids from micelles has directly proven to release GLP-1 and GIP via the FFAR1 in the duodenum (264).

This release of bile acids, mainly cholic (CA) and chenodeoxycholic (CDCA) acid derivatives, happens through the relaxation of the smooth muscle sphincter upon CCK signaling (336) or indirectly through a similar VIP action on the sphincter of Oddi (337).

Historically described as mere fat-solubilizing agents, these amphipathic compounds were recently recognized as key signaling molecules capable to modulate the host metabolism directly acting as ligands of intestinal GPCRs (101, 338, 339), or after being metabolized by the colonic microbiota into secondary bile acids, mostly deoxycholic and lithocholic acid(340).

The chemosensor believed to be the main receptor of bile acids is TGR5, also known as GPR131 or GPBAR1 among other names. This receptor has been reported to be expressed by colonic GLP-1-secreting enteroendocrine cells and pancreatic α-and β-cells (100, 101), with some controversy regarding the presence in murine islets (339).

TGR5 activity appears to not have been lost in type 2 diabetic humans whereby the infusion of CCK, or rectal taurocholate, causes GLP-1 and insulin release via the TGR5 axis in colonic L-cells and pancreatic β-cells respectively (341, 342).

This notion is in stark contrast to the well-known anti-diabetic properties of BAs sequestrants, (343) and some, have proven to elicit GLP-1 secretion via TGR5 mediated PC1/3 upregulation (344). A likely explanation is that the BAs bound to a sequestrant into the intestinal lumen can't be absorbed and hence travel more distally in the GI tract where the complexes are still capable to activate the TGR5 expressing colonic L-cells. Furthermore, the lower systemic levels of bile salts prompt the liver to produce more bile, which in turn feeds more TGR5 agonism into the colon (343).

This chemosensor is expressed by the pancreatic α-cells where its signaling activates Gs proteins and induces the secretion of GLP-1 directly through Epac proteins and indirectly via CREB mediated expression of Psck1, while in β-cells mediates insulin release [(100); **Figure 3**].

TGR5 is the target of different BAs, but the most potent endogenous agonist has shown to be lithocholic acid (LCA) and its taurine conjugates with activity at nanomolar concentrations (273, 339). Secondary bile salts, metabolized by the microbiota, exhibit less potency toward this receptor.

Despite this promising anti-diabetic activity of TGR5 mediate by GLP-1 (345), its pharmacological activation in diabetic patients has shown side effects at the level of gallbladder and heart, hampering its clinical use (346).

Another bile salts chemosensor is the nuclear farnesoid X receptor (FXR) (347) which activation, contrarily to TGR5, blocks the release of GLP-1 in the colonic L-cells (348), while in the liver induces glycogenesis helping to improve glucose homeostasis. This counterintuitive pharmacology has been confirmed *in vivo* whereby the administration of the FXR agonist GW4064 by mouth drives hyperglycaemia and obesity (349) while intraperitoneal injection exerts protection from it (350). Consistently, an indirect inhibition of intestinal FXR through microbiota modulation, or genetic deletion of intestinal FXR, corroborate this phenome displaying protection from high-fat diets induced obesity and fatty liver disease (351).

This could explain why bile acid sequestrants support a positive glucometabolic homeostasis. Indeed, the insoluble complexes of bile salts can activate lumen-facing TGR5 receptors, while they cannot cross plasma membranes to activate intracellular GLP-1-suppressant FXR receptors.

FXR is a very important receptor, part of a negative feedback in the liver, whereby the binding of bile salts, especially

chenodeoxycholic acid, represses the *de-novo* synthesis of bile salts (352, 353). Indeed, there are multiple primary or secondary bile acid chemosensors in the liver (348, 354) or scattered along the gastrointestinal tract (355), where they ensure a direct negative feedback aiding detoxification (356) and protecting from hepatotoxicity and carcinogenicity displayed by some secondary bile salt such as lithocholic acid.

Accumulated evidence, indicate how bile acids are important modulators of the whole body metabolism, bridging the microbiome to the brain, likely being key signaling molecules in the pathogenesis of obesity and type 2 diabetes. Indeed remittance from diabetes experienced by RYGB or SG patients, has been attributed to the elevation of circulating bile acids (37, 38, 357), warranting further investigation, especially the development of gut-restricted TGR5 agonists (358).

TRPV1 AND THE TRP CHANNEL FAMILY

The transient receptor potential vanilloid 1 (TRPV1) is a tetrameric non-specific cationic channel found in most of mammalian sensory neurons (359). Each of its constituting monomers crosses the plasma-membrane six times and both the N and C-term face the cytoplasmic side, where they make up 70% of the receptors' entire volume (360). This chemosensor, together with other 27 non-selective cationic channels, is part of a larger family named transient receptor potential (TRP) channel superfamily and is known to play an important role in the metabolic syndrome (361, 362).

TRPV1 is primarily activated by vanilloids and capsaicinoids including Capsaicin (360), eliciting the sensation of spiciness; multiple stress-related stimuli cause its activation and opening with subsequent membrane depolarization. For example cigarette smoke, excess of protons (pH < 5.9) (363), temperatures above 43° (360), certain animal toxins (364, 365), ATP (366) or even cannabinoids such as Anandamide (367) and cannabidiol (359, 368), are all stimuli known to activate this sensor. Indirect stimulation has also been demonstrated by bradykinin (366), NGF (366), PGE2 (369), PGI2 (369) and agonists of Protease-activated Receptors (PARs) (370).

TRPV1 has been shown to be expressed in the brain, β-cells (371), nociceptor C fibers, dorsal root ganglia, hepatocytes, spermatozoa (372), airway neurons (373), bladder and urothelium (374), blood vessels, and the whole gastrointestinal myenteric plexus (375), especially in colonic and rectal neurons (376). Consistently, TRPV1 is also found to be expressed by the murine enteroendocrine cell line model STC-1 and its agonism induces the release of GLP-1 *in vivo* (377).

This receptor has recently seen an increasing interest since its activation has been found to have pleiotropic beneficial metabolic effects (378).

Indeed, it has been known for more than a decade that capsaicin is capable to elicit a glucose-stimulated insulin release *in vivo* (379). A crossover study operated on 30 human healthy subjects (380), showed a slight increase in plasmatic GLP-1 and a slight decrease in ghrelin levels 30 min after a Capsaicin enriched meal (containing 1,030 mg of 80,000 Scoville heat units red pepper); Peptide YY changes were not statistically significant. Despite these promising results, TRPV1 knockout mice display contrasting phenotypes with the report of opposite phenotypes. One author describes an obese insulin and leptin resistant mouse (381), while another group report animal protected from diet-induced obesity (382).

Considering all the recent findings, drugs targeting TRPV1 would be beneficial for the management of obesity (383) metabolic syndrome (384) and type 2 diabetes (385). Nonetheless, considering the EECs receptome responsible for gut-peptide modulation, TRPV1 has received much less attention, with a yet largely unexplored physiology.

THE MICROBIOTA

Animals' GI tract is known to host a population of hundreds of different species of bacteria (386), viruses and fungi, estimated to equal in number the cells that constitute the human body (387). These microorganisms thrive in the colon's lumen, where they secrete small molecules ultimately affecting the host immunity (240) and metabolism (388).

The relative abundance of different microbial species is known to depend on the presence of specific nutrients (389); hence, considering that an imbalance in the microbiota correlates with chronic inflammation pathologies of the bowel, or even Type 2 diabetes, it is likely that dietary components indirectly influence the occurrence of these pathologies via the microbiota (390, 391).

The human colonic microflora is known to produce high concentrations of Short-Chain-Fatty acids (SCFAs), among other metabolites, from the anaerobic fermentation of dietary indigestible carbohydrates, or even derivatives of bile salts (389). In fact, the SCFAs Acetate, Propionate and Butyrate are the principal luminal anions in humans and other mammalian's colon (309, 392), with some inter-species variability. Rats show higher levels of fecal Acetate, 75 mM vs. human's 50 mM, Propionate, 27 vs. 11 mM and Butyrate, 16 vs. 5 mM respectively. On the other hand, surprisingly similarly to humans' colonic and fecal values, rumens of herbivores, such as sheep or cows, also contain high levels of acetate, propionate and butyrate, with reported concentrations of 65, 21, and 18 mM, respectively (308). These levels appear to be independent of dietary proteins or fibers; conversely, it is the caloric intake that affects the relative composition and concentrations of SCFAs (308). These metabolites have been found to target specific receptors among the repertoire expressed by the EECs, triggering a hormonal response. It is estimated that in humans almost all fermented SCFA are absorbed by the colonocytes and only 5% are excreted with stool, equivalent to 5–30 millimoles per day. Indeed, it is not practically feasible to measure intraluminal production fluxes of various metabolites *in vivo* in humans; therefore, most studies focus on the easiest but less informative quantification of fecal SCFA content (393).

Despite the most recent studies of transgenic and germ-free animals, it is still largely unknown by what degree hormones such as GLP-1, and all its related peptides, depend on the microflora, especially in pathologies such as type 2 diabetes.

Recent high-throughput pharmacogenomic studies have deepened our understanding of the molecular players in this human-microbiota relationship. Recently it was shown that a new class of N-acyl amides is produced by the microbiota, and target GPCRs expressed by the enteroendocrine cells, modulating GLP-1 expression and overall glucose metabolism. In particular, N-oleoyl serinol (N-OS) is described as a potent GPR119 agonist, acting in the lower micromolar range with twice the efficacy of the endogenous ligand OEA (394).

From the evolutionary perspective, dietary components, together with the microbiota-fermented products, have activated the enteroendocrine system for billions of years, since the dawn of metazoan. Considering the vast and continuous pool of metabolites produced and modulated by the microbiota, the distinction between orthosteric and allosteric ligand becomes blurred; different molecules are likely working in synergy to elicit a specific hormonal response.

Modulation of the microbiome has shown promising results in the treatment of type 2 diabetes. For example, recently it was reported that a rhubarb extract, Rhein, increasing the intestinal population of Bacteroidetes, mediates an increase in ileal GLP-1 producing cells, peripheral GLP-1(7-36)$_{NH2}$ levels and improved glucose tolerance in diabetic db/db mice (395). Consistently, STZ-treated rats, are protected from oxidative and inflammatory stress when treated with Liraglutide, and *Bacteroides*, as well as *Lactobacilli* strain populations appear to be restored (396).

In the last decade, the scientific community has just started to unveil the molecular pathways produced by this long-lasting symbiosis. It appears that SCFAs not only induce the release of GLP-1, they also represent a mitogenic signal. Rats fed oligofructose, a substrate for the colonic microbiota which leads to higher SCFAs levels, possess an increased number of colonic L-cells (397). This has been confirmed *ex-vivo* in human and mouse small intestinal crypts organoids (398).

Other compounds such as bile salts and xenobiotics (399), are known to be metabolized and excreted by the microbiota, affecting the host physiology. Indeed, the pharmacokinetic and pharmacodynamics of any drug taken by mouth should be appraised considering the role of the microbiota, as the varied efficacy of some chemotherapeutics such as 5-FU has been proven to directly depend on this host-microbiota metabolism (400). Even though the anatomical intestinal rearrangement of RYGB and SG patients is known to affect the microbiota, this doesn't appear to result in a different bile acid metabolism in a rat model (401).

We are at the beginning of a new branch of medical practice, tailored not only to the single person genome, but also to the microbiome.

Future human studies will help us to better understand the big picture of this relationship, to hopefully provide mechanistic knowledge upon which new treatments could be created, such as microbiome-directed gene-therapies for the management of metabolic diseases.

CONCLUSION AND PERSPECTIVE

GLP-1R-independent signaling of GLP-1, its intra-islet axis, and its once-thought inactive metabolites, all represent new important additions to our understanding of this peptide in health and disease.

Omnivores' gastrointestinal tract has co-evolved in strict relationship with a dynamic microbiota and a complex seasonal and regional diet, resulting into a robust and flexible system tightly interconnected via multiple neuroendocrine axes with different organs.

In nature, dietary fats are scarce energy-dense nutrients primarily found in fish and meat. This evolutionary pressure over millions of years has shaped a system for the attentive sensation, assimilation and storage of precious bioactive molecules in all superior animals.

Sensation happens at multiple levels with a plethora of somewhat redundant intestinal receptors (402), specifically in the enteroendocrine cell system. This redundancy can be seen in transgenic animals, whereby the genetic absence of a single chemosensor doesn't always result in a phenotype, probably due to metabolic compensation from similar and overlapping pathways.

Virtually all macronutrients are absorbed in the small intestine, where maximal activity of the EECs is ensured, while the colonic and rectal GLP-1 secretion is enforced in response to secondary metabolites even hours after the meal ingestion. This pattern is disrupted in bariatric patients undergoing RYGB surgery, where a remodeled GI tract delivers more nutrients to the large intestine, and changes gut-secretome, including its microflora.

Attempts to mimic this altered meal processing, such as proximal blockage of nutrient absorption resulting in increased delivery of nutrients in the distal intestine, have shown some promising results in healthy and diabetic humans (403). Although this is more challenging with fats because dietary lipids require partial digestion by lipases to become efficient secretagogues (404, 405). However, distant delivery of free fatty acids, or even Oleoyl-Glycerol and sodium taurocholate have shown negligible effects on peripheral levels of GLP-1 or PYY, satiety and glucose tolerance (311, 406, 407). Similarly, distal delivery of the best known aminoacidic GLP-1-secretagogue, glutamine, has proven ineffective at ameliorating glucose tolerance in both healthy and diabetic subjects (407–409).

Furthermore, a recent report (410) examined the effect of RYGB on lean pigs, and indicates how it is the post-operative GLP-1 (9-36)$_{NH2}$ levels that raise, while surprisingly the "active" (7-36)$_{NH2}$ peripheral levels were reduced.

Indeed, most authors focus only on the peripheral levels of only one of these two peptide species, vastly excluding GLP-1(28-36) $_{NH2}$ and (32-36)$_{NH2}$ activity, rendering the overall understanding of each individual GLP-1 species, in both health and disease, difficult to discern.

Technical advances ELISA, capable to specifically dissect these peptide species locally and peripherally, will help us to shed new light into this complex physiology (411).

Conclusively, bearing in mind that insulinotropic or incretinotropic effects are not secondary to any single receptor modulation, whereby pools of different luminal stimuli act synergistically on tens of different chemosensors during their intestinal transit and absorption, while interacting with the microflora metabolism, rendering the restoration of a healthy

physiology in diabetic patients with the pharmacological correction of a single axis, highly improbable.

The final dissection of the molecular axis causative of either metabolic syndrome will need more evidence regarding the localized and inter-neuronal physiology of GLP-1 in physiological and pathological statuses. To ultimately tease apart any possible cause from secondary events, species-specific biology will also need to be carefully dissected and interpreted.

AUTHOR CONTRIBUTIONS

SP researched and interpreted all the data from available scientific literature on the PUBMED database, organized, wrote and revised the whole manuscript. SP also conceptualized and drew all the figures assembling the final formatted review. MF conceived, organized, wrote and revised the whole manuscript.

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REVIEW
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Role of Intestinal Bitter Sensing in Enteroendocrine Hormone Secretion and Metabolic Control

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The gastrointestinal tract stores ingested nutrients in the stomach which are then delivered to the small intestine at a controlled rate to optimize their digestion and absorption. The interaction of nutrients with the small and large intestine generates feedback that slows gastric emptying, induces satiation, and reduces postprandial glycemic excursions. The mechanisms underlying these nutrient-gut interactions are complex; it has only recently been appreciated that the gut has the capacity to detect intraluminal contents in much the same way as the tongue, via activation of specific G-protein-coupled receptors, and that ensuing signaling mechanisms modulate the release of an array of gut hormones that influence gastrointestinal motility, appetite and glycemia. Interestingly, evidence from preclinical models supports a functional link between intestinal bitter taste receptor (BTRs) and gastrointestinal hormone secretion, and the outcomes of recent studies indicate that stimulation of intestinal BTRs may be used to modulate gastrointestinal function, to diminish energy intake and limit postprandial blood glucose excursions in humans. This review summarizes current evidence about the expression and function of intestinal BTRs in relation to enteroendocrine hormone release and discusses the clinical implications of this pathway for the management of obesity and type 2 diabetes.

Keywords: bitter taste receptors, gut hormones, enteroendocrine cells, energy intake, blood glucose, obesity, type 2 diabetes

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INTRODUCTION

Recent decades have witnessed the conceptual evolution of the gastrointestinal tract from being solely a site of nutrient digestion and absorption to its recognition as the largest endocrine system in the body - more than 30 peptides are now known to be released from enteroendocrine cells within the gastrointestinal mucosa. These gut-derived hormones communicate with tissues both within and outside the gut, and play a pivotal role in the regulation of metabolic homeostasis. Of particular importance are ghrelin, released from the enteroendocrine Gr-cells (within the stomach); cholecystokinin (CCK), from I-cells (mainly in the upper small intestine); glucose-dependent insulinotropic polypeptide (GIP), from K-cells (largely in the upper small intestine); and glucagon-like pepetide-1 (GLP-1) and peptide YY (PYY), from L-cells (predominantly in the distal small and large intestine) (**Figure 1**). Ghrelin is secreted predominantly during fasting and is suppressed after

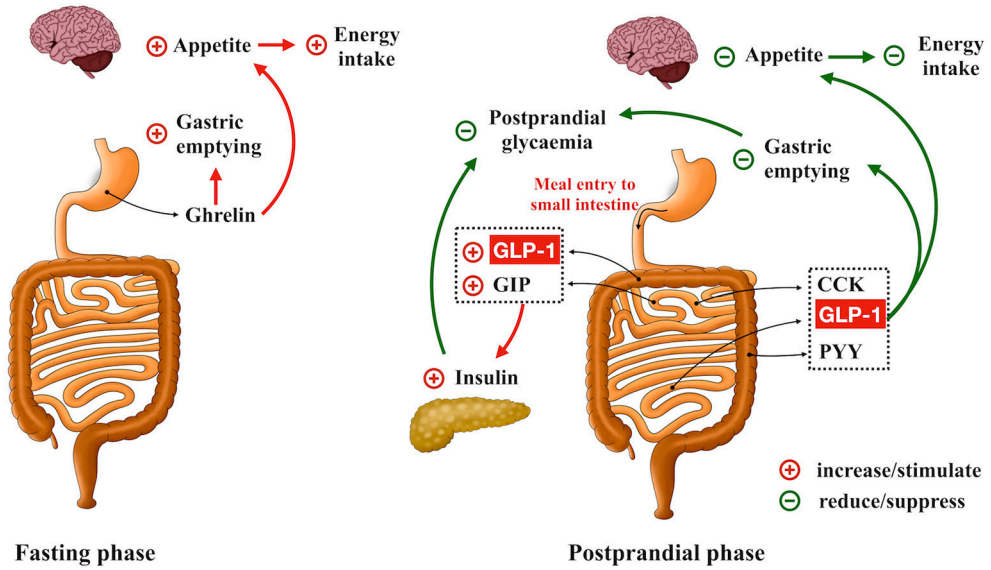

FIGURE 1 | Role of gastrointestinal hormones in the regulation of gastric emptying, postprandial glycemia and energy intake. Ghrelin is secreted during fasting and acts to accelerate gastric emptying, promote appetite and drive energy intake. GLP-1, GIP, CCK, and PYY are released in the postprandial phase. GLP-1 and GIP are the 'incretin' hormones, stimulating insulin secretion in a glucose-dependent manner. GLP-1, CCK, and PYY also form intestinal feedback to slow gastric emptying and suppress energy intake.

meals. It is regarded as a "hunger" hormone that drives food intake and accelerates gastric emptying (1, 2). In contrast, CCK, GIP, GLP-1, and PYY are predominately released postprandially and, in concert, mediate intestinal feedback to limit postprandial glycemic excursions and suppress energy intake (2, 3). In health, GIP and GLP-1 are responsible for the substantially greater insulin response to oral, or enteral, glucose administration when compared with "isoglycaemic" intravenous glucose infusion–the so-called "incretin" effect (4). In type 2 diabetes, the insulinotropic effect of GLP-1 remains relatively intact, although that of GIP is markedly diminished, which may account for the diminished incretin effect in this group (5). GLP-1 also exerts a glucose-dependent glucagonostatic effect (5) and, together with CCK and PYY, acts to slow gastric emptying and suppress energy intake (2). Accordingly, modulation of gut hormone secretion has been actively pursued as a therapeutic option in the management of obesity and type 2 diabetes (5–12). To this end, it has been suggested that a wide array of chemo-sensors expressed on different enteroendocrine cells is responsible for the detection of carbohydrate [e.g., ATP-sensitive K^+ channel and sodium glucose co-transporter-1 (13, 14)], fat [e.g., G-protein-coupled receptors 119 and 120 (15, 16)] and protein [e.g., oligopeptide transporter 1 and calcium sensing receptor (17, 18)] and associated stimulation of gut hormone secretion. Emerging evidence also attests to the functional importance of "taste" signals arising from intraluminal contents in modulating gut hormone release. For example, blockade of intestinal sweet taste receptors (STRs) by lactisole attenuates glucose-induced incretin hormone secretion substantially in healthy humans (19), although stimulation of STRs (by low-calorie sweeteners) alone appears insufficient to stimulate GIP or GLP-1 secretion in humans (20). Unlike STRs, activation of intestinal bitter taste receptors (BTRs), either by pharmacological BTR agonists or physiological bitter compounds, has been shown to modulate gut hormone secretion in various preclinical and clinical experimental settings, leading to reductions in blood glucose and energy intake (21, 22). In this review, we summarize current evidence relating to the expression and function of intestinal BTRs in relation to enteroendocrine hormone release, as well as the clinical implications of this pathway for the management of obesity and type 2 diabetes.

INTESTINAL BITTER TASTE RECEPTORS

Taste stimuli are detected by a group of specialized G protein-coupled receptors, initially identified in the taste buds of the oral cavity (23). Subtypes of taste 1 receptors heterodimerize to detect sweet (T1R2/T1R3) and umami (T1R1/T1R3) stimuli, while multiple type 2 receptors (T2Rs) are characterized as BTRs and detect bitter stimuli, and may trigger mechanisms which prevent the ingestion and absorption of potentially noxious bitter compounds. Binding of ligands to these taste receptors initiates a signaling cascade involving the dissociation of the G-protein gustducin into Gα and Gβγ subunits, activation of phospholipase C β_2, production of diacylglycerol and inositol 1,4,5-trisphophate (21, 24, 25), and opening of the transient receptor potential ion channel M5, leading to the release of intracellular Ca^{2+} (21, 24, 26–28), Na^+ influx (26, 29), cellular depolarization and the secretion of neurotransmitters (28). The increases in intracellular Gα subunit also activate phosphodiesterase to degrade cyclic adenosine monophosphate (cAMP), whereas diacylglycerol and

intracellular Ca^{2+} activate the protein kinase C pathway (21, 26) (**Figure 2**). It has only recently been appreciated that taste receptors and their downstream signaling molecules are also found in extra-oral locations, including the airway, kidney, brain, immune system and the gastrointestinal tract (30, 31). For example, in rodents, inhalation of BTR agonists decreases airway resistance (32), while intravenous administration of the BTR agonist, denatonium benzoate (DB), causes a transient fall in blood pressure (33). The focus of this review, however, is the biology of intestinal BTRs, and in particular their relevance to the secretion of gastrointestinal hormones from enteroendocrine cells.

In a seminal study reported in 2002, Wu et al. demonstrated gene expression of several T2Rs in both the stomach and duodenum of mice and rats using reverse transcriptase-PCR (34). In addition, T2Rs were also found to be expressed on the secretin tumor cell line (STC-1), an enteroendocrine cell model derived from murine enteroendocrine tumors (34). That the exposure of STC-1 to different bitter compounds resulted in a rapid increase in intracellular Ca^{2+} indicated that a functional BTR-sensing system may be present on the enteroendocrine cells (34). These observations were further validated in subsequent studies employing reverse transcriptase- and quantitative-PCR assays on small and large intestinal tissues and enteroendocrine cells of both rodents and humans (**Table 1**) (25, 42, 43). Consistent with PCR observation, studies using double-labeling immunofluorescence have also shown co-localization of chromogranin A (a cellular marker of enteroendocrine cells) with T2Rs in the mouse small and large intestine (42, 44). More specifically, co-expression of GLP-1 with various T2Rs in human enteroendocrine L cell lines (i.e., HuTu-80 and NCI-h716) and in small and large intestinal tissues has been observed (21, 35, 36, 39). However, the co-expression of T2Rs with enteroendocrine cells containing other hormones is not well characterized in rodents or humans. Moreover, the expression of intestinal BTRs in metabolic disorders has not been consistently reported. In the study reported by Chao et al. (49), the expression of both STR and BTR subtypes were shown to be less in the hypothalamus, brainstem and duodenum in ob/ob mice than C57Bl/6 controls. By contrast, the expression of the BTR, T2R38, in the colonic mucosa was shown to be related directly to BMI in humans, such that the abundance of T2R38 tended to be higher in those who were overweight/obese, when compared to lean subjects (40). In both healthy individuals and patients with type 2 diabetes, the expression of STRs in duodenal biopsy samples did not correlate with BMI or HbA1c, although the dynamic response of STR expression to intraduodenal glucose infusion was found to be impaired in type 2 diabetes (50). Of note, the downstream signaling molecules of taste receptors have also been identified in non-endocrine cells of the gut. For example, α-gustducin and transient receptor potential ion channel M5 are expressed abundantly in subsets of brush cells in mouse and rat gut (51–53). In murine gastric tissue, α-gustducin-expressing brush cells have been found adjacent to ghrelin-releasing Gr-cells (54, 55). Given that the latter are not in direct contact with the intraluminal contents, i.e., "closed-type," it is possible that brush cells may act as a sensor for intraluminal contents to regulate ghrelin secretion (56).

EFFECTS OF BTR SIGNALING ON GUT HORMONE SECRETION

An increasing number of studies in both preclinical and clinical models have evaluated the effects of BTR agonists on ghrelin, CCK, GLP-1, and PYY secretion, although the specificity of bitter compounds for different T2Rs is poorly defined and the function of intestinal BTR sensing in either obesity or type 2 diabetes has not been thoroughly investigated. In contrast, information regarding GIP secretion in response to BTR stimulation is limited (**Table 2**).

Ghrelin

The potential role of BTR signaling in the regulation of ghrelin secretion has evaluated in mice and humans, albeit with strikingly different outcomes. In mice, intragastric administration of a mixture of BTR agonists (including DB, phenylthiocarbamide (PTC), quinine and D-[-]salicin) was shown to increase plasma total ghrelin and octanoyl ghrelin levels without affecting ghrelin mRNA expression (55). BTR agonist-induced ghrelin secretion was markedly attenuated in α-gustducin-/- mice. This was consistent with a functional involvement of taste signaling in ghrelin release (55), although α-gustducin is a non-specific downstream signaling molecule and, as discussed, an indirect interaction between brush cells and Gr cells is an alternative possibility. Paradoxically, intragastric gavage of BTR agonists in mice was associated with only a transient increase in food intake during the first 30 min, followed by a sustained suppression of intake over the subsequent 4 h (55). In contrast to the stimulation of ghrelin observed in mice, intragastric administration of another bitter tastant, quinine-hydrochloride (HCl quinine, 10 umol/kg), reduced fasting plasma ghrelin and motilin levels in healthy women (22, 63), associated with increased activity in hedonic and homeostatic brain regions on functional magnetic resonance imaging, and suppressed antral motility and energy intake (22). These observations suggest a role of BTR signaling in communications between the gut and brain in the control of energy intake. However, in another study, intragastric DB at a dose of 1 umol/kg, which suppressed motilin secretion, appetite scores and energy intake, failed to affect either plasma ghrelin or the rate of gastric emptying in healthy women (57). Accordingly, further studies are required to determine the secretory pattern of ghrelin in response to different types and doses of BTR agonists and the associated metabolic effects in humans, including those with obesity and type 2 diabetes.

CCK

Initial evidence to support the potential for BTR-evoked CCK secretion was reported in STC-1 cells, where both DB and PTC increased intracellular Ca^{2+} and stimulated CCK secretion in a dose-dependent manner (43, 61). Subsequently, steroid glycoside H.g.-12, extracted from the plant *Hoodia gordonii* [which tastes bitter, and has potent appetite-suppressant effects in both animals and humans (64)] was found to induce CCK

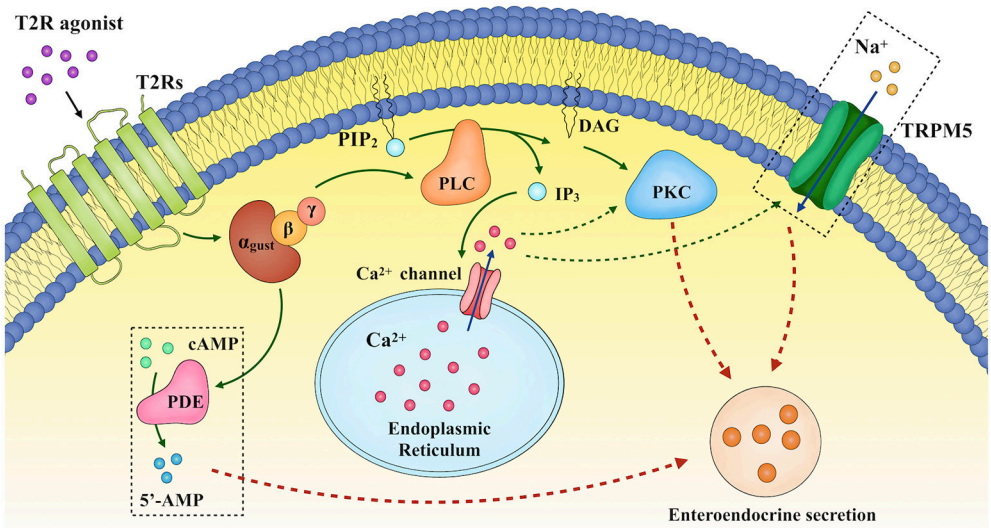

FIGURE 2 | Proposed mechanisms underlying enteroendocrine secretion in response to T2R agonists**.** Binding of ligands to bitter taste receptors (BTRs) triggers a signaling cascade involving the dissociation of the G-protein gustducin into Gα and Gβγ subunits, activation of phospholipase C β_2 (PLCβ_2), production of diacylglycerol (DAG) and inositol 1,4,5-trisphophate (IP_3), and opening of the transient receptor potential ion channel M5 (TRPM5), thereby leading to the release of intracellular Ca^{2+} ($[Ca^{2+}]_i$), Na^+ influx, cellular depolarization and the secretion of neurotransmitters. DAG and $[Ca^{2+}]_i$ also activate the protein kinase C (PKC) pathway. In addition, increases in intracellular Gα subunit activate phosphodiesterase.

TABLE 1 | Summary of published reports on the presence of different T2Rs in enteroendocrine cells and gastrointestinal tissues in rodents and humans.

Species	Models	T2Rs expressed	References
Human	HuTu-80 cell	T2R4, T2R5, T2R13, T2R14, T2R16, T2R38, T2R39, T2R40, T2R44, T2R46, T2R47, T2R49, T2R50, T2R60	(35–37)
	NCI-H716 cell	T2R1, T2R3, T2R4, T2R5, T2R7, T2R8, T2R9, T2R10, T2R13, T2R14, T2R19, T2R20, T2R30, T2R38, T2R39, T2R40, T2R41, T2R45, T2R46, T2R50, T2R60	(21, 24, 38, 39)
	Small intestine	T2R5 T2R14 T2R38	(36, 37, 39)
	Large intestine	T2R1, T2R3, T2R4, T2R5, T2R10, T2R13, T2R38, T2R39, T2R40, T2R42, T2R43, T2R44, T2R45, T2R46, T2R47, T2R49, T2R50, T2R60	(35, 36, 38, 40, 41)
Mouse	STC-1 cells	mT2R102, mT2R104, mT2R105, mT2R106, mT2R107, mT2R108, mT2R109, mT2R110, mT2R113, mT2R114, mT2R116, mT2R117, mT2R118, mT2R119, mT2R121, mT2R122, mT2R123, mT2R124, mT2R125, mT2R126, mT2R129, mT2R130, mT2R131, mT2R134, mT2R135, mT2R136, mT2R137, mT2R138, mT2R139, mT2R140, mT2R143, mT2R144	(25, 42, 43)
	Small intestine	mT2R102, mT2R104, mT2R105, mT2R106, mT2R107, mT2R108, mT2R110, mT2R113, mT2R114, mT2R116, mT2R117, mT2R119, mT2R121, mT2R122, mT2R123, mT2R124, mT2R126, mT2R129, mT2R130, mT2R134, mT2R135, mT2R136, mT2R137, mT2R138, mT2R139, mT2R140, mT2R143, mT2R144	(26, 44–47)
	Large intestine	mT2R108, mT2R113, mT2R117, mT2R118, mT2R119, mT2R125, mT2R126, mT2R131, mT2R135, mT2R136 mT2R137, mT2R138, mT2R140, mT2R143	(26, 46–48)
Rat	Small intestine	rT2R1, rT2R2, rT2R3, rT2R4, rT2R5, rT2R6, rT2R7, rT2R8, rT2R9, rT2R10, rT2R12, rT2R16, rT2R34, rT2R38	(34, 48)
	Large intestine	rT2R, rT2R16, rT2R26	(41)

secretion both *ex vivo* from rat intestine, and from HuTu-80 cells (37). That the effect of H.g.-12 on CCK secretion was abolished by a BTR inhibitor, compound 03A3, supports a functional role of BTR signaling in H.g.-12-induced CCK release (37). While co-expression of BTRs with CCK-secreting I-cells has not been assessed in humans, oral administration of encapsulated HCl quinine (18 mg) was recently reported to increase plasma CCK concentrations and reduce energy intake at an *ad libitum* meal in healthy young individuals (62). Moreover, in this study the magnitude of suppression of energy intake in response to HCl quinine was related directly to the subjects' sensitivity to the bitter taste of PTC (62). These observations warrant further investigation on the potential of targeting the intestinal BTR signaling pathway

TABLE 2 | Effects of bitter tastants on gut hormone secretion in preclinical and clinical models.

Hormone	Preclinical/Clinical	*Vitro*/*Vivo*	Model	Bitter tastants	References
Ghrelin	Preclinical	*vivo*	Mice	Mixture of DB, quinine, PTC, D-salicin	(55)
			Human	HCl quinine 10 umol/kg	(57)
	Clinical			HCl quinine 10 umol/kg	(22)
GLP-1	Preclinical	*vitro*	HuTu-80 cells	Phenylthiourea	(36)
			NCI-716 cells	Berberine	(24)
				1,10-phenanthroline	(39)
				Gentiana scabra	(58)
				DB	(21)
			STC-1 cells	extract from wild bitter gourd	(59)
				Berberine	(25)
		vivo	Mice	Extract from wild bitter gourd	(59)
				DB	(21)
				Qing-Hua Granule	(29)
				Gentiana scabra	(58)
	Clinical		Healthy volunteer	Gentiana lutea root	(60)
CCK	Preclinical	*vitro*	STC-1 cells	DB and PTC	(43)
			HuTu-80 cells	H.g.−12 (extract of the plant Hoodia gordonii)	(37)
			Caco-2 cells	PTC	(61)
		vivo	mice	Mixture of DB, quinine, PTC, D-salicin	(61)
	Clinical		healthy volunteer	HCl quinine 10 mg	(62)
PYY	Preclinical	*vitro*	NCI-716 cells	DB	(21)

to stimulate CCK secretion and reduce energy intake in obesity.

GLP-1 and PYY

Underpinned by the successful clinical application of GLP-1 receptor agonists and dipeptidyl peptisase-4 inhibitors to the management of type 2 diabetes (5, 11, 12), there has been great interest in the potential for BTR agonists to augment L-cell secretion, and thereby increase concentrations of endogenous GLP-1.

At the cellular level, numerous bitter compounds have been reported to induce GLP-1 secretion from enteroendocrine cells via BTR pathways. For example, in both NCI-716 and STC-1 cells, berberine, a natural bitter plant alkaloid commonly used as an antibiotic, was shown to dose-dependently stimulate GLP-1 secretion via T2R38 (24, 25). Similarly, a specific T2R38 agonist, phenylthiourea, induced GLP-1 secretion from HuTu-80 cells, an effect markedly inhibited by silencing of T2R38 with small interfering RNA (36), In contrast, 1,10-phenanthroline stimulates GLP-1 via T2R5 (39), and DB appears to induce GLP-1 secretion via a broad range of BTRs (including T2R4, T2R43, and T2R46 at least), in NCI-h716 cells (21). Furthermore, blockade of BTRs (e.g., by probenecid), or the downstream pathways relating to BTR signaling, including inositol 1,4,5-trisphophate, phospholipase C β_2, protein kinase C and/or phosphodiesterase, attenuates GLP-1 secretion induced by bitter tastants (21, 58, 59).

In rodents, exposure of the gut to BTR agonists has also been shown to augment plasma GLP-1 levels (21, 36, 58, 59). In acute settings, an intragastric preload of DB prior to enteral glucose administration increased plasma GLP-1 and insulin concentrations (21), slowed gastric emptying (26, 65) and reduced blood glucose (21). Consistent with the role of BTR signaling in GLP-1 secretion, the effect of DB to slow gastric emptying was abolished by co-administration of probenecid (26). Similarly, intragastric administration of PTC has been reported to augment plasma GLP-1 concentrations (36) and slow gastric emptying (26) in mice. The latter effect was, however, not inhibited by probenecid (26). This discrepancy necessitates further investigation to determine whether probenecid sufficiently blocks the BTRs activated by PTC, and whether mechanisms other than BTR-gut hormone pathways account for the slowing of gastric emptying by PTC in mice. In support of the latter, the slowing of gastric emptying induced by a mixture of bitter substances (including PTC) was not affected by concurrent administration of GLP-1 and CCK antagonists in mice (55). In the longer-term (i.e., 4 weeks), intragastric administration of DB remained effective at increasing meal-induced GLP-1 secretion, associated with a reduction in body weight in obese mice, whereas another bitter tastant, quinine, had minimal effect on GLP-1 or ghrelin, despite reducing body weight (66).

While BTRs (e.g., T2R5 and T2R38) have been reported to localize on L-cells in the small and/or large intestine, effects of BTR agonists on GLP-1 secretion are not well characterized in humans. Recently, Mennella et al. evaluated the effect of a single low dose of *Gentiana lutea* root extract encapsulated for release in the small intestine in healthy subjects (60), and observed a tendency for a higher GLP-1 response to a standardized breakfast, and a reduction in post-lunch energy intake compared to placebo (60). Accordingly, additional human studies are needed to

evaluate the potential for targeting intestinal BTRs to stimulate GLP-1 secretion.

In contrast to GLP-1, information relating to the effect of BTR agonists on PYY secretion (also released from L-cells) is limited. Although DB stimulates PYY secretion from NCI-H716 cells in a similar manner to GLP-1 (21), this effect has hitherto not been assessed *in vivo*.

CLINICAL IMPLICATIONS OF TARGETING INTESTINAL BTRS

That BTR signaling is functionally linked to the secretion of hormones integral to the regulation of energy intake and glycemia, as well as the control of gastric emptying, has stimulated substantial interest in targeting this pathway for the management of obesity and type 2 diabetes (publications from clinical studies are summarized in **Table 3**). The relative absence of calories in bitter compounds represents a substantial asset of this approach.

Effects on Energy Intake

The impact of BTR sensing in the control of energy intake has been evaluated in both preclinical and clinical studies. Despite variable effects of different BTR agonists on each gastrointestinal hormone, the majority of studies in rodents have reported energy intake to be suppressed following exposure to acute doses of BTR agonists (69–71), although one study reported a transient increase, followed by a sustained suppression of food intake after intragastric administration of a mixture of DB, PTC and salicin (55). Arguably, of greater interest is evidence that intragastric gavage of DB (60 μmol/kg) or quinine (160 μmol/kg) once daily for 4 weeks in high fat-fed obese mice reduced weight gain substantially, and in an α-gustducin-dependent manner (66). In healthy women, a single dose of HCl quinine (10 umol/kg), administrated intragastrically 60 min before an *ad libitum* liquid meal (chocolate milk shake), reduced food intake (346 ± 37 g for HCl quinine vs. 414 ± 46 g for water control), in association with reduced ghrelin levels and increased neural activity in the hypothalamus, hedonic regions, and parts of the medulla associated with appetite homeostasis (22). Consistent with these observations, oral administration of encapsulated HCl quinine (18 mg) also modestly suppressed energy intake at a subsequent *ad libitum* buffet meal (514 ± 248 kcal for HCl quinine vs. 596 ± 286 kcal for placebo) in healthy young subjects (12 females and 8 males) without inducing nausea (62). Likewise, administration of encapsulated bitter compounds derived from *Gentiana lutea* root with a standardized breakfast reduced total daily energy intake by ∼20% in healthy individuals (60), while oral insensitivity to the bitter taste of 6-n-propylthrouracil was associated with increased energy intake in female subjects (72). It remains to be determined whether stimulation of intestinal BTRs has the capacity to reduce energy intake and, hence, body weight in obese individuals.

Effects on Blood Glucose

The rate of emptying of carbohydrates from the stomach for absorption in the small intestine is a major determinant of the glycemic response to meals (73). In the majority of type 2 diabetic patients with modestly elevated glycated hemoglobin (HbA1c < ∼8% or 64 mmol/mol), postprandial glycemia makes the dominant contribution to overall glycemic control (74, 75). In addition, postprandial glycemia is an independent cardiovascular risk factor and predicts all-cause mortality (76), and accordingly, represents a specific target for the treatment of type 2 diabetes. Preclinical models indicate that stimulating intestinal BTRs has the potential to improve blood glucose control. In wild type mice, intragastric administration of DB, PTC or a mixture of bitter compounds slowed gastric emptying substantially (26, 55), while oral administration of DB (1 mg/kg) (21) or *Gentia scabra* root extract (300 mg/kg; containing several bitter compounds such as loganic acid, gentiopicrin and rindoside) (21, 58) in db/db mice was associated with higher GLP-1 and lower blood glucose responses following glucose gavage when compared with saline. In mice fed a high fat diet, oral administration of bitter gourd extract prior to an oral or intraperitoneal glucose load also resulted in higher GLP-1 and insulin levels and lower blood glucose responses (59). That the magnitude of reduction in glycemia was attenuated substantially by concurrent administration of the GLP-1 receptor antagonist, exendin(9–34, 36, 39, 42–44), attests to the importance of GLP-1 to glucose-lowering induced by bitter substances (59).

Hitherto, there is limited information about the effect of BTR agonists on blood glucose in humans. Studies to date have reported inconsistent effects on gastric emptying. In healthy women, sham-feeding with quinine sulfate (10 mg) was reported to slow the emptying of subsequently ingested "electrolyte soup," when compared to sham-feeding with a "pleasant" strawberry flavoring or control (no sham-feeding) (67). Little et al. compared the rate of gastric emptying of three "test meals" in healthy subjects, consisting of 500 mL water (control) and two bitter-tasting solutions containing either a small dose of quinine (1 mM) or naringin (0.198 mM), delivered via intragastric infusion. Although these doses of quinine and naringin yielded a medium intensity of bitterness during an oral perception test, gastric emptying did not differ between the bitter solutions and water alone (68). More recently, intragastric administration of DB at a dose of 1 umol/kg suppressed appetite sensations, but failed to affect gastric emptying in healthy women (68). However, it remains unclear whether the disparity in findings between studies in mice and humans reflect species differences, or whether the relatively low doses of BTR agonists employed in the human studies were insufficient to interact with L-cells located predominantly in the distal small and large intestine. In the case of GLP-1, infusion of glucose into the duodenum at 2 kcal/min (where glucose is absorbed in the upper gut) elicits minimal GLP-1 secretion, while ileal infusion of glucose at the same rate induces substantial GLP-1 release (77).

The genetic phenotype of GPCRs is now known to be an important determinant of physiological function, may predispose to human diseases (78). There is evidence that polymorphisms of BTR genes that impair the sensitivity to bitterness may be associated with changes in food intake and dysregulation of blood glucose. For example, women with gestational diabetes mellitus exhibited a lower T2R9 gene (rs3741845) frequency, and consumed more meat, dairy and sweet beverages compared

TABLE 3 | Effects of bitter tastants in clinical studies.

Authors	Subjects	Bitter tastants and doses	Main method	Key observation
(67)	healthy women ($n = 16$)	10 mg quinine sulfate	Sham feeding	Slowed gastric emptying substantially.
(68)	healthy volunteers ($n = 12$)	0.198 mM 500 ml quinine (3.24 mg)	Intragastric administration	Had no effect on gastric emptying.
(62)	healthy volunteers ($n = 20$)	18 mg HCl quinine	encapsulated	Suppressed energy intake; increased CCK secretion; had no effect on gastric emptying.
(60)	healthy volunteers ($n = 20$)	100 mg extracts (from Gentiana lutea root)	encapsulated	Increased GLP-1; suppressed energy intake; had no effect on blood glucose.
(57)	healthy women ($n = 39$)	1 μmol/kg DB	Intragastric administration	Had no effect on gastric emptying; reduced hungry rating and increased satiety ratings.
(63)	healthy women ($n = 10$)	10 μmol/kg HCl quinine	Intragastric administration	Reduced plasma motilin and ghrelin levels; inhibited the antral motility.
(22)	healthy women ($n = 16$)	10 μmol/kg HCl quinine	Intragastric administration	Suppressed energy intake; reduced plasma motilin and ghrelin levels; reduced hungry ratings and increased satiety ratings.

to pregnant women without gestational diabetes mellitus (79). Similarly, dysfunction of T2R9 due to a single nucleotide polymorphism is associated with higher blood glucose and insulin responses to an oral glucose tolerance test in Amish individuals with and without type 2 diabetes (38). In German individuals without type 2 diabetes, variations in the T2R38 gene (rs713598, rs1726866 and rs10246939) are also reported to have significant associations with body composition in women, and the glycemic response to oral glucose in men (80).

CONCLUSIONS AND PROSPECTIVE VIEWS

In recognition of the pleiotropic actions of gastrointestinal hormones in the regulation of metabolic homeostasis, exogenous peptides or mimetics (e.g., GLP-1 receptor agonists and GLP-1/GIP dual receptor agonists) are under rapid development within the pharmaceutical industry to better manage both type 2 diabetes and obesity. This approach, however, is often limited by cost, side effects (predominantly gastrointestinal symptoms), and suboptimal efficacy (particularly for obesity). Dietary strategies to modulate endogenous gastrointestinal hormone secretion represent an alternative that shows substantial promise. For example, consuming a nutrient 'preload' prior to the main meal has been shown to reduce postprandial blood glucose in both health and type 2 diabetes by stimulating GLP-1 secretion in advance of the meal, and by slowing gastric emptying (10, 81, 82). However, this approach entails additional energy intake associated with the preload. Modulation of gastrointestinal hormone secretion by low- or non-caloric compounds, such as bitter tastants, would therefore be advantageous compared with nutrient preloads.

There is a large body of preclinical studies that provide compelling evidence of a functional BTR signaling system in enteroendocrine cells, the effects of non-nutritive BTR agonists on enteroendocrine hormone secretion, and the potential for stimulating intestinal BTRs to suppress energy intake and reduce postprandial glycemic excursions (59, 66). However, there are only a handful of clinical studies in healthy subjects (mostly females) that have evaluated the effects of BTR signaling on gut hormone secretion and associated metabolic effects, and no studies in patients with obesity and/or type 2 diabetes. Moreover, the doses of BTR agonists administered in human subjects have been low, probably because bitter tastants are considered to be potentially toxic and aversive (28). Bitter taste perception in the mouth is unpleasant, and naturally serves as an aversive signal for the termination of eating. However, stimulation of intestinal BTRs by administration of different BTR agonists directly into the stomach or duodenum, thereby bypassing oral perception, has not been reported to cause any adverse effects in preclinical models and healthy subjects. Nevertheless, the tolerability of BTR agonists at higher doses remains to be established.

Relative to STRs (T1R2/T1R3) and umami taste receptors (T1R1/T1R3), the biology of BTRs (T2Rs) appears to be more complex due to their diversity. Moreover, expression of BTRs varies substantially along the gastrointestinal tract. For example, T2R2 and T2R6 showed higher expression in gastric than duodenal mucosa in rats (34), whereas in mice, T2R118 and T2R131 are expressed abundantly in the colon, but minimally in the duodenum and jejunum (46). As summarized in **Table 1**, multiple T2Rs are often co-expressed on the same enteroendocrine cell. However, the relative importance of each has not been characterized. Accordingly, it remains to be determined whether the expression of T2Rs also exhibits regional specificity, in a similar pattern to enteroendocrine cells and, therefore, whether more targeted delivery of BTR agonists is needed for effective stimulation of enteroendocrine hormone secretion. Notably, physiological bitter substances, including bile acids and products of digestion (e.g., amino acids), are abundantly present in the gut after a meal; it

is also important, therefore, to understand the physiological role of intestinal bitter taste sensing in the regulation of gastrointestinal hormone secretion, appetite and postprandial glycemia.

AUTHOR CONTRIBUTIONS

CX, XW, RY, MH, CR and TW were all involved in conception, design and writing of the manuscript. All authors have approved the publication of this final version of the manuscript.

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The Origin and Understanding of the Incretin Concept

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Gastrointestinal hormones that stimulate insulin secretion at physiological concentrations are incretins. This concept has recently attracted considerable attention in the wake of drugs developed from the gut hormone GLP-1 (glucagon-like peptide-1) for diabetes therapy. But the renewed enthusiasm has also restricted the concept to just two hormones, GLP-1 and GIP (glucose-dependent insulinotropic polypeptide). The purpose of the present overview is two-fold: First to tell that the incretin concept is far from new. It has a more than a century long history full of ups and downs. Second, that the incretin concept may now have become too narrow. Thus, it is likely that incretin comprises additional gastrointestinal hormones, which interact with GIP and GLP-1 during normal meals containing protein, fat and complex carbohydrates (and not just pure glucose). Such broader incretin concept may stimulate development of novel gut hormone-derived drugs.

Keywords: gastrointestinal hormones, incretin, GIP, GLP-1, history of incretin

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INTRODUCTION

In gastrointestinal endocrinology, the concept of incretin is today highly topical and generally applied to two distinct gut hormones with technical acronymous names: GIP (originally "gastric inhibitory polypeptide," later renamed "glucose-dependent insulinotropic polypeptide") and GLP-1 ["glucagon-like peptide 1," now in its truncated (7–36) form]. Of course it is more idiomatic to use the single word "incretin" instead of two acronyms. Moreover, many younger scientists and physicians today consider incretin a rather novel and fashionable concept surfacing in the wake of the development of GLP-1-derived drugs for treatment of type 2 diabetes mellitus, a view furthered by the growing business for the diabetes-related pharma industry. In other words, there is at present a marked focus on the two above-mentioned hormones in gastrointestinal endocrinology. To this end, there are also articles which are ostensibly dealing with gut hormones but mainly report about GLP-1 and/or GIP, sometimes accompanied by measurements of a third gut hormone, PYY ("peptide tyrosyl-tyrosyl"), or measurements of ghrelin [see for instance (1–4)]. These articles contribute to the picture that gastrointestinal endocrinology today is essentially about GIP and GLP-1.

It is exciting that gut hormones are now used as targets for development of drugs for major diseases with large numbers of patients. But this is in fact what has been attempted for more than a century. Likewise, the incretin concept as such is in some respects more than 140 years old. But with the present conceptualization to just two hormones, incretin may lose aspects of its meaning and understanding of what gastrointestinal endocrinology is fundamentally about. Equally unfortunate, initiatives to develop additional relevant drugs may also be lost with today's narrow view on incretin.

In this situation, a review on the origin and early phases of gastrointestinal endocrinology leading to the incretin concept may be pertinent. The report here may hopefully also pave the way for a fuller and more relevant understanding of the biology of gut hormones, and at the same time give due credit to pioneers in the incretin story.

DEFINITION OF INCRETIN

Incretin is a word and concept constructed for a gut hormonal factor assumed to supplement secretin in the effect on pancreatic secretion. Thus, while secretin stimulates the secretion of water and bicarbonate from the *exocrine* pancreatic cells (5, 6), it has been suggested from the beginning that (an) other gut hormone(s) would stimulate the internal or *endocrine* secretion from pancreatic islet-cells (6, 7). Literally, it was the Belgian physiologist Jean La Barre who coined the word "incrétine" in 1932 (8). Consequently, the original definition suggests that any gut hormone which under physiological circumstances stimulates or contributes to the stimulation of the secretion of pancreatic hormones [insulin, glucagon, PP (pancreatic polypeptide), and pancreatic somatostatin] is an incretin.

The physiological context is of course important. Since the function of hormones in the digestive tract fundamentally is to facilitate digestion and subsequent absorption and metabolism of food elements, the incretin activity is linked to the gastrointestinal processing of ordinary meals. Hence, the original incretin definition challenges unphysiological loadings, such as intake of large amounts of for instance pure glucose or other pure chemicals.

THE HISTORY OF INCRETIN

1850–1900: The mental framework for the idea of incretin dates back to the second half of the nineteenth century, where European physiologists began to focus on the mechanisms of the external and internal secretion of the pancreas (5, 9–13). It was in this period that Mering and Minkowski showed that the pancreas was the site of origin for diabetes mellitus (14), and where Claude Bernard tried to explain the fact that significantly larger amounts of glucose can be given orally than intravenously without glucosuria. Hence, Claude Bernard suggested that the liver takes up most of the oral glucose during the first portal circulation in order to prevent hyperglycemia (15). This explanation had supporters up to the 1950's (16).

1900–1960: A decisive breakthrough came in 1902, with Bayliss' and Starling's hallmark discovery of secretin (5, 6) that founded not only gastrointestinal endocrinology, but also endocrinology in general. The discovery of secretin was also the background for Starling's Croonian lecture from 1905 (6), in which he coined the word *hormone* (from Greek "hormoa": I arouse to activity). According to Moore et al. (7), the discovery of the first hormone, secretin, also led Starling to suggest the possibility that the duodenal mucosa in addition to secretin produces another hormone that stimulates the internal secretion of the pancreas. Moore et al. immediately tested Starling's hypothesis by oral administration of extracts of duodenal mucosa to three recently diagnosed diabetes patients (7). In hindsight, the results of such oral intake were of course negative and inconclusive, because protein- and peptide-hormones are proteolytically degraded in the stomach. But the idea of incretin was born more than 110 years ago.

After the Banting & Best discovery of insulin in 1922 (17), new attempts were taken to examine extracts of the duodenal mucosa and their influence on blood glucose concentrations (18–21). These results were also conflicting and inconclusive (21), which in retrospect cannot surprise. But promising results were nevertheless obtained by La Barre and Still, who in 1930 reported that they in the *in vitro* processing of duodenal extracts had obtained two interesting fractions: One with crude secretin, which in sophisticated cross-circulation experiments in dogs stimulated the secretion from the exocrine pancreas, and another which lowered blood glucose concentrations without effect on the secretion of the exocrine pancreas (22). They also suggested that the glucose-lowering effect was due to stimulated insulin secretion (22). Then in 1932—as mentioned above (8)—La Barre presented the name, incretin, together with suggestions for treatment of diabetes mellitus with incretin (23). Strictly speaking, the articulated idea of incretin-therapy for diabetes is thus nearly a century old.

After La Barre's hallmark contributions to the incretin story (8, 21–23), the Austrian Hans Heller also prepared an extract of the duodenal mucosa, which he in 1935 reported to lower blood glucose concentrations—even after oral administration to rabbits and man (24). Heller named the active factor in his extract "duodenin." His results, however, have not been followed up, and the unspecific name duodenin was rapidly forgotten. Then, synchronously with the onset of the Second World War, the incretin-idea in general suffered an almost deadly blow from the Chicago-school of gastrointestinal endocrinology (25–27). The school was founded by Andrew Ivy, well known from the discovery of cholecystokinin (CCK) in 1928 (28). After three publications in rapid succession 1939–40 about acidification of the duodenum in dogs at various blood glucose concentrations (25–27), Ivy et al. concluded that the existence of an incretin is unlikely. This opinion was neither challenged nor contradicted during World War II and in the two first post-war decades. On the contrary, the younger Ivy-pupil and -successor as spokesman for American gastrointestinal endocrinology, Morton Grossman, emphasized in a comprehensive high-impact review in 1950 the scepticism against the incretin concept (29). But as time has shown, Ivy and co-workers were wrong. They drew false-negative conclusions of their experiments that in fact only showed that secretin in dogs is without significant effect on insulin secretion. Nevertheless, their publications paralyzed further ideas and initiatives about incretin for a quarter of a century.

1960–2000: The year 1960 witnessed a major breakthrough for biomedicine and not least endocrinology. It virtually changed the world and revitalized the interest in incretin. It was the invention of the radioimmunoassay (RIA) by Berson and Yalow (30). The RIA technique allowed for the first time in a fairly uncomplicated, but accurate manner measurement of molecules present in pico- to even femtomolar concentrations. A world of biologically active

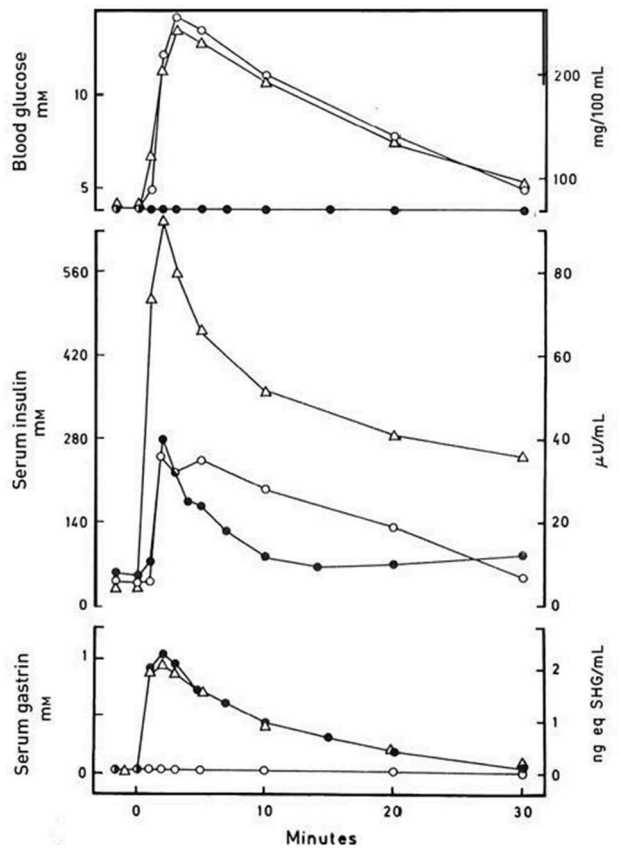

FIGURE 1 | Blood glucose, serum insulin and serum gastrin concentrations in a normal subject after i.v. injection of synthetic human gastrin-17 (SHG), 250 ng kg^{-1} body weight (•), after i.v. injection of 25 g glucose (o), and after a synchronous i.v. injection of both 250 ng kg^{-1} gastrin-17 and 25 g glucose (Δ). Data from Rehfeld & Stadil (44).

TABLE 1 | Twelve milestones in the first century of the history of the incretin concept.

Name(s)	Contribution	Year	References
1. Mering and Minkowski	Pancreas as the site of diabetes	1889	(14)
2. Bayliss and Starling	Discovery of secretin; the first hormone	1902	(5)
3. Starling	A gut hormone may stimulate the endocrine pancreas	1905	(6)
4. La Barre and Still	Evidence of an insulinotropic gut hormone	1930	(22)
5. La Barre	Coining the word incretin	1932	(8)
6. Yalow and Berson	Invention of the radioimmunoassay	1960	(30)
7. McIntyre et al. and Elrick et al.	Demonstration of a glucose-dependent incretin mechanism	1964	(33, 34)
8. Unger et al.	Gut glucagon-like immunoreactivity	1966	(52)
9. Brown et al.	Identification of GIP	1971	(46, 47)
10. Dupré and Brown	GIP as an incretin	1973	(48)
11. Bell et al.	Identification of GLP-1	1983	(55, 56)
12. Habener et al. and Holst et al.	Truncated GLP-1 as an incretin	1987	(59, 60)

substances, including peptide hormones, circulates in plasma in those concentrations. Therefore, RIA methods expanded the dimensions of much biological and medical research. Not least in basic and clinical endocrinology, because hormones are defined by their circulation in blood. For good reasons, the RIA technology was first applied to insulin (30). Therefore, the method was immediately embraced by endocrinologists and diabetologists studying pancreatic endocrine secretion and diabetes mellitus. Only one year later, RIA measurement of glucagon was launched by Unger et al. (31). And each year during the following decades bursted with novel RIAs for known and new pancreatic and gastrointestinal hormones (32).

The possibility of direct and reliable measurements of insulin in plasma soon reopened the incretin question. In 1964, laboratories in London, UK [McIntyre et al. (33)] and Denver, US [Elrick et al. (34)] independently showed that oral glucose provokes a considerably larger insulin response than intravenous glucose, even at similar blood glucose concentrations. Hence, the gut harbors indeed insulinotropic hormonal factors. Or in other words, the incretin mechanism exists. The reports of McIntyre and Elrick et al. catalyzed new incretin studies in man, which followed three lines.

One line focussed on *in vivo* development of methods for quantitation of the glucose-induced incretin effect. Here, Perley and Kipnis (35) showed that the incretin part of the insulin response to oral glucose in man constituted more than half, later confirmed to be probably two thirds or more of the insulin response in healthy people, though smaller, with high age and some gastrointestinal diseases (36, 37). Another line examined the incretin effect of the then known troika of gastrointestinal hormones (secretin, gastrin, and CCK) that could be obtained in more or less pure forms in the mid-1960's (38–43). These studies were later reinvestigated with pure, synthetic peptides (44, 45). The immediate results of the studies were less encouraging. Oral glucose only elicited modest (gastrin and CCK) or no increase (secretin) of endogenous secretion of the known gut hormones. And the effect of isolated exogenous administration in physiologically relevant doses of for instance gastrin only stimulated insulin secretion to a minor extent [(44), see also **Figure 1**]. A third line obtained more success with two later identified gut hormones. In the early 1970's, first in the laboratory of Viktor Mutt in Stockholm, John Brown isolated GIP as an inhibitor of gastric acid secretion (46, 47). In subsequent studies, however, John Brown together with John Dupré showed that GIP is a potent releaser of insulin during hyperglycemia, but without effect in euglycemia (48). Thus, GIP was a glucose-dependent incretin and was accordingly renamed "glucose-dependent insulintropic polypeptide," hence maintaining the acronym with the new name [for reviews, see also Creutzfeldt (49) and (50)]. The following quantitative studies of the incretin

TABLE 2 | Examples of gastrointestinal neuroendocrine peptides that require (further) examination of their incretin activity*.

Adrenomedullin
Apelin
Calcitonin Gene-Related Peptide (CGRP)
Cholecystokinin
Galanin
Gastrin
Gastrin-Releasing Peptide (GRP)
Ghrelin
Leptin
Motilin
Neurotensin
Neuropeptide Y (NPY)
Obestatin
Opioids
Pituitary Adenylate Cyclase Polypeptide (PACAP)
Peptide YY (PYY)
Vasoactive Intestinal Polypeptide (VIP)
Xenin

**Full examination requires studies of the isolated effect on basal and stimulated islet-hormone secretion as well as studies of synergistic effects in combination with the other gastrointestinal hormones (including GIP and GLP-1).*

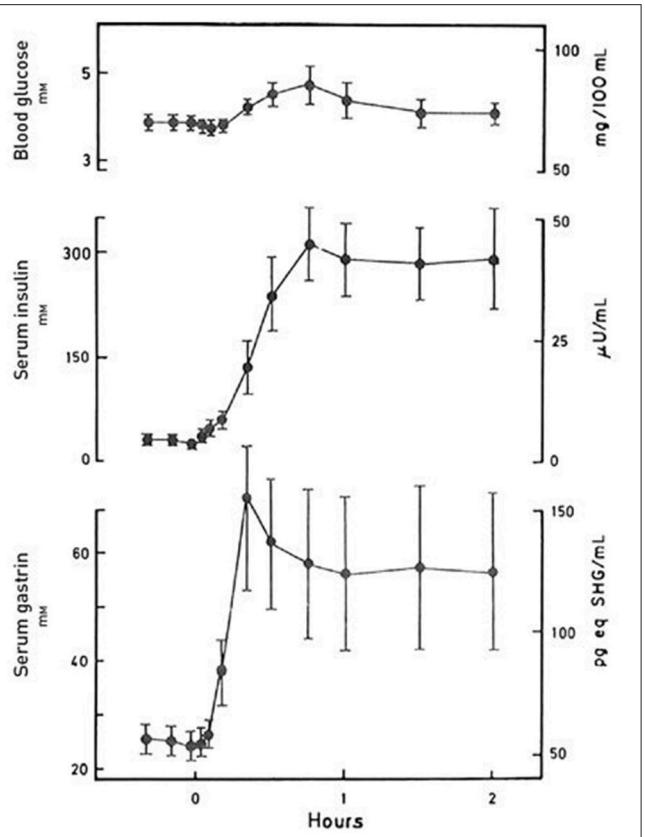

FIGURE 2 | Blood glucose, serum insulin and serum gastrin concentrations during a protein-rich meal. The concentrations are indicated as mean ± SEM ($n = 8$). Data from Rehfeld & Stadil (44).

effect of GIP, however, suggested that GIP could not explain the entire gut hormonal effect on insulin secretion after oral glucose.

Then in the mid- and late 1980's, an additional gut hormone with incretin-activity surfaced. The background for the discovery was Unger and co-workers' RIA-observations in the 1960's that the intestinal mucosa expressed some glucagon-like immunoreactivity, which was different from the well-known pancreatic glucagon peptide; hence the name "gut glucagon" (51–53). Several laboratories in Europe and North America subsequently tried to identify the bioactivity and structure of gut glucagon peptides in the hope that one of them might be a missing incretin [for review, see for instance (54)]. An essential premise for success in this endeavor became the cloning and sequencing of mammalian glucagon genes by Graeme Bell and co-workers in 1983 (55, 56). The cDNA-deduced proglucagon structure revealed unequivocally that the prohormone in addition to the sequence of pancreatic glucagon contained the sequences of two novel *g*lucagon-*l*ike *p*eptides, which by Bell et al. were named GLP-1 and GLP-2. Both GLP's were expressed in the gut. GLP-1 as such had a modest insulin-releasing activity (57), but purification from gut extracts in the laboratories of Habener and Holst, respectively, showed that GLP-1 was also synthetized in a truncated (7-36) form with marked insulin-releasing effect (58–61). Moreover, the truncated GLP-1 turned out also to inhibit the secretion of pancreatic glucagon, which together with its insulinotropic effect (59, 60) counteracts the hyperglycemia in diabetes (62, 63). That GLP-1 moreover is a satiety signal that facilitates weight loss and— as shown later—ameliorates the cardiac function in diabetes has made GLP-1 an obvious drug target for treatment of type 2 diabetes mellitus. Several GLP-1-derived drugs are consequently now on the market and have been subject to comprehensive randomized and controlled trials [for recent reports, see (64–68)]. So far, so good for GLP-1 and GIP as incretins (2, 4). Essential milestones in first century of the history of the incretin concept are pinpointed in **Table 1**.

A PROBLEM

While nobody questions the insulinotropic activities of GIP and GLP-1, it has become a problem that the present enthusiasm for the two hormones and not least for GLP-1-derived drugs has virtually suppressed supplementary ideas about additional incretin activity of other gut hormones. The problem reflects an old-fashioned and somewhat incorrect view on gastrointestinal endocrinology, not least among GLP-1 enthusiasts. There are different aspects to consider in this context.

First, the original definition of incretin is as stated "any gut hormone, which under physiological circumstances stimulates the secretion of pancreatic hormones." Indeed, several gastrointestinal hormones beyond GIP and GLP-1 stimulate insulin (see **Table 2**). For instance, gastrin accentuates glucose-stimulated insulin secretion significantly (**Figure 1**), and occasionally also glucagon secretion (37, 44). The effect of the

other hormones administered exogenously alone in the fasting state may, however, be small and look trivial. But in combination with for instance EGF (epidermal growth factor), GLP-1 and/or during a meal (**Figure 2**), the effect may be significant as discussed in detail for instance for gastrin and cholecystokinin (37, 44, 69–73). Also the new gut hormone, xenin (74) displays promising incretin activities (75–77). And acute administration of PYY (1-36) as well as somatostatin inhibits insulin secretion. Moreover, examination of gut hormone receptors on the cell-membranes of pancreatic islet-cells is likely to show that a considerable number of gastrointestinal hormones directly influence the secretion of pancreatic hormones. For instance, Reubi et al. found a fairly abundant expression of gastrin and CCK receptors on human pancreatic islet cells (78).

Second, the old textbook-understanding of gastrointestinal endocrinology has been a "one-hormone-one-target" without functional overlap between the hormones: Gastrin regulated gastric acid secretion; secretin pancreatic bicarbonate secretion; CCK mainly gallbladder emptying; GIP only inhibition of gastric secretion; motilin intestinal motility etc. This understanding has in many ways turned out to be wrong and misleading. Today we know that the digestive tract is the largest and phylogenetically oldest endocrine organ in the body in which 30 different hormone genes are expressed and where the prohormones are cellularly processed to more than 100 bioactive peptides. Each hormone system has several targets both in and outside the gastrointestinal tract. And different hormones may simultaneously target the same organ and cells synergistically with both stimulatory and inhibitory signals. Moreover, the same enteroendocrine cell may express two or more different hormone genes. And the enteroendocrine cells for a given hormone are considerably more widespread in the gut than hitherto assumed. For instance gastrin/CCK_2-receptor agonists are expressed all the way from the stomach to colorectal mucosa [for reviews, see for instance (79, 80)]. Thus, the limitation of incretin activity to only two peptides from the gastrointestinal tract may be somewhat naïve and old-fashioned.

Third, the delineation of incretin activity in such close relation to intake of glucose is also problematic. Of course, the concentrations of glucose in circulation are relevant in studies and discussions of insulin and glucagon secretion. But oral intake of 50 or 75 g pure glucose as used in the oral glucose tolerance tests is an unphysiological situation, which cannot be used to exclude gut hormones as incretins under normal physiological conditions. Several gut hormones respond as mentioned poorly to pure glucose. But many respond vividly to normal meals containing substantial amounts of protein, fat, and complex carbohydrates without major changes in blood glucose concentrations and under these circumstances stimulate islet-hormone secretion in synergy with other gut hormones (**Figure 2**). This in fact touches the fundamental role of the enteroendocrine system: That meals—depending on their composition—elicit variable polyphonies or rather symphonies of gastrointestinal hormones playing together to ensure optimal digestion and absorption of the food. This is the situation that is relevant for definition of general endocrine activities of the gut. Not only regarding incretin activity, but also other major cross-hormonal activities such as gastrointestinal motility, the inhibitory gastrone activities, satiety signaling etc., where several different hormones interact for the purpose of ensuring optimal nutrition.

CONCLUSION

Incretin and the use of incretin hormones in diabetes therapy are old concepts with roots dating back to the second half of the nineteenth century. The history of incretin reflects a development characteristic for many lines of science with alternating progress and retrogressions. The situation for incretin today is based on decisive technical breakthroughs in disciplines such as peptide purification and sequencing; radioimmunoassay technology; cDNA cloning and sequencing; *in vitro* perfusion of endocrine organs; and *in vitro* synthesis of polypeptide constructs containing even three agonist epitopes. Probably, continued incretin research will reveal further integration of GIP and GLP-1 with additional gut peptides and provide a better and more comprehensive physiological understanding of the incretin concept. Such understanding may further the development of biomedical diagnosis and incretin therapy. This development is in fact already underway both in terms of GIP, GLP-1, and/or glucagon dual and triple receptor agonists (81–84), and—perhaps even more promising—dual or triple receptor agonists that combine GLP-1 analogs with analogs of some of the other gastrointestinal hormones (85–87).

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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