Molecular mechanisms underlying physiological and receptor pleiotropic effects mediated by GLP-1R activation.

Abbreviated title: Molecular Physiology of GLP-1R

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Summary
The incidence of type 2 diabetes in developed countries is increasing yearly with a significant negative impact on patient quality of life and an enormous burden on the healthcare system. Current biguanide and thiazolidinedione treatments for type 2 diabetes have a number of clinical limitations, the most serious long term limitation being the eventual need for insulin replacement therapy. Since 2007, drugs targeting the glucagon like peptide 1 (GLP-1) receptor have been marketed for the treatment of type 2 diabetes. These drugs have enjoyed a great deal of success even though our underlying understanding of the mechanisms for their pleiotropic effects remain poorly characterised even while major pharmaceutical companies actively pursue small molecule alternatives. Coupling of the GLP-1 receptor to more than one signalling pathway (pleiotropic signalling) can result in ligand dependent signalling bias and for a peptide receptor such as the GLP-1 receptor this can be exaggerated with the use of small molecule agonists. Better consideration of receptor signalling pleiotropy will be necessary future drug development. This is particularly important given the recent failure of taspoglutide, the report of increased risk of pancreatitis associated with GLP-1 mimetics and the observed clinical differences between liraglutide, exenatide and the newly developed long-acting exenatide long acting release, albigitide and dulaglutide.

Key words
Type 2 diabetes, glucagon like 1 receptor, GLP-1, GLP-1R, GPCR, G protein-coupled receptor

Abbreviations
ADP, adenosine diphosphate; ANS, autonomic nervous system; ATP, adenosine triphosphate; Bad, Bcl-2-associated death promoter; cAMP, cyclic adenosine monophosphate; CREB, cAMP response element binding protein; DPPIV, dipeptidyl
The incretin effect

The observation that oral glucose administration results in significantly higher pancreatic insulin secretion compared with intravenous dosing to the same plasma concentration gave rise to the idea of an entero-insular axis that was responsible for this enhancement (Elrick et al., 1964; Mcintyre et al., 1964). The implication being that there was glucose sensing within the gastrointestinal tract or hepatic system that resulted in potentiated insulin secretion, called the incretin effect, a concept dating back to the late 19th century (reviewed in (Creutzfeldt, 1979)). It was shown that two gut-derived hormones gastric inhibitory polypeptide (GIP (Dupre et al., 1973)) and glucagon like peptide 1 (GLP-1 (Kreymann et al., 1987)), known as incretins, were secreted in response to meal ingestion and could stimulate insulin secretion, thereby making a significant contribution to overall
postprandial insulin release. Both GIP and GLP-1 have no effect on insulin secretion in the absence of elevated plasma glucose obviating the risk of hypoglycaemia (Nauck et al., 1993a). However, the insulinotropic effects of GIP are lost in type 2 diabetics (T2DM) (Nauck et al., 1993b), while these effects from GLP-1 are maintained, leaving GLP-1 as the primary candidate incretin for clinical use.

The insulinotropic action of GLP-1 appears to be entirely mediated by the GLP-1 receptor (GLP-1R) (Scrocchi et al., 1996) (we note that the official NC-IUPHAR nomenclature (Alexander et al., 2011) for this receptor is GLP-1 and have used GLP-1R to aid distinction between ligand and receptor). Activation of the GLP-1R on pancreatic β-islets results in potentiation of glucose dependent insulin secretion as well as improvements in β-cell function and mass in animal models (Xu et al., 1999; Perfetti et al., 2000; Stoffers et al., 2000; Tourrel et al., 2001; Farilla et al., 2002; Rolin et al., 2002; Wang and Brubaker, 2002; Bock et al., 2003; Sturis et al., 2003; Gedulin et al., 2005), although some of this effect may be mediated centrally and will be discussed later. Its activation forms the basis of two classes of glucose lowering agents, incretin mimetics (i.e. GLP-1 receptor agonists) and inhibitors of dipeptidyl peptidase IV (DPPIV or CD26)(incretin enhancers), an enzyme responsible for cleavage of GLP-1 to inert metabolites.

**Biology of GLP-1 synthesis, secretion and metabolism**

Tissue specific post-translational cleavage of proglucagon generates glucagon in pancreatic α-cells (Patzelt et al., 1979) and GLP-1, GLP-2, oxyntomodulin and glicentin in intestinal L-cells (Orskov et al., 1987; Eissele et al., 1992) and the brain (Drucker and Asa, 1988; Larsen et al., 1997). GLP-1(1-37) is processed in intestinal L-cells into two equipotent circulating molecular forms GLP-1(7-36) amide and GLP-1(7-37) (Holst et al., 1987;
Kreymann et al., 1987; Mojsov et al., 1987). In humans, GLP-1(7-36) amide represents the majority of circulating active GLP-1 secreted in response to nutrient ingestion (Orskov et al., 1994) and will henceforth referred to simply as GLP-1. GLP-1 is secreted in a biphasic pattern with early phase beginning within 5-15 min and prolonged second phase observed from 30-60 min of meal ingestion (Herrmann et al., 1995). GLP-1 is rapidly cleaved between positions 8 and 9 by the widely expressed serine protease DPPIV into GLP-1(9-36)amide (and GLP-1(9-37)) giving a circulation half life of 2-3 minutes (Mentlein et al., 1993b; Deacon et al., 1995; Kieffer et al., 1995; Hansen et al., 1999). These DPPIV processed GLP-1 peptides have low in vitro affinity and activity at the classically defined human GLP-1R (Knudsen and Pridal, 1996; Montrose-Rafizadeh et al., 1997a) but have been shown to have cardioprotective and glucoregulatory actions when pharmacologically dosed (Nikolaidis et al., 2005; Meier et al., 2006; Elahi et al., 2008) and, in model animals, may exert effects independent of GLP-1R (Ban et al., 2008; 2010). The relevance of the pharmacological effects of these metabolites is unclear, particularly given they are subject to rapid renal clearance with a half-life of less than 5 minutes (Ruiz-Grande et al., 1993; Meier et al., 2004).

GLP-1 is also a substrate for the metalloendopeptidase enzyme neprylisin, also known as neutral endopeptidase 24.11 (Hupe-Sodmann et al., 1995). A single study in the pig demonstrated that neprylisin is responsible for degrading almost half the circulating GLP-1 (Plamboeck et al., 2005), consistent with findings that the GLP-1 analogue liraglutide is also degraded by neprylsin in humans (Malm-Erjefält et al., 2010). Neprylisin cleaves GLP-1 at a number of positions (Hupe-Sodmann et al., 1995) and although it is theorised that these products could have mitochondrial effects (Tomas and Habener, 2010), their physiological concentrations make this unlikely.

As mentioned above, oxyntomodulin (OXM) is also a product of tissue specific cleavage of proglucagon. OXM consists of amino acids 33-69 of proglucagon and is a
cleavage product of glicentin containing the full 29 amino acids of glucagon with an 8 amino-acid carboxy terminal extension (for a comprehensive review see (Holst, 2007)). In both intestinal L-cells and cells of the nucleus of the solitary tract in the brain processing of proglucagon generates GLP-1, GLP-2 and glicentin which is only partly processed into OXM (Mojsov et al., 1986; Orskov et al., 1987; Larsen et al., 1997). In the intestinal L-cells all these peptides are co-secreted in response to food intake (reviewed in (Pocai, 2012)). OXM is a low potency full agonist for cAMP accumulation from the GLP-1R (Schepp et al., 1996; Jorgensen et al., 2007) and the glucagon receptor (GCGR) (Jorgensen et al., 2007). It is also a full agonist for recruitment of G protein-coupled receptor kinase (GRK) 2, β-arrestin1, and β-arrestin 2 to the GCGR, however at the GLP-1R, OXM is only a partial agonist for these interactions (Jorgensen et al., 2007). Despite this OXM has a higher affinity for the GLP-1R than GCGR and thus, GLP-1R is proposed as the primary receptor for this peptide.

**The glucagon like peptide 1 receptor; structure & expression**

In humans, GLP-1R is a 463 amino acid G protein-coupled receptor (GPCR) belonging to the secretin-like family (also referred to as Family B). This is a small family of only 15 GPCRs including receptors for secretin, GIP and vasoactive intestinal peptide (VIP). Structural characteristics of this receptor family include: a relatively long, extracellular N-terminal domain responsible for high affinity binding of endogenous peptide ligands; 6 highly conserved cysteine residues in the extracellular domain that form 3 conserved disulphide bridges; an amino terminal signal peptide, several N-linked glycosylation sites and of course the characteristic seven transmembrane bundle shared by all GPCRs (reviewed in (Furness et al., 2012)).

The sites of GLP-1R expression in both model organisms and humans have been investigated using a variety of techniques of varying sensitivity and resolution. Potential roles
for the receptor in physiological processes that are regulated by its ligands may be predicted by its location. In many cases there are significant shortcomings in the molecular identification of sites of expression of the GLP-1R and in these cases better molecular and functional data would be invaluable.

Expression of the GLP-1R has been demonstrated in pancreatic islets of rodents and humans (Orskov and Poulsen, 1991; Campos et al., 1994; Körner et al., 2007), which is consistent with the large amount of data demonstrating GLP-1 potentiation of glucose stimulated insulin secretion (GSIS). Insulin secreting β-cells comprise 65-80% of the cells of the pancreatic islet with glucagon secreting α-cells comprising 15-20% and somatostatin secreting δ-cells 3-10% (reviewed in (In’t Veld and Marichal, 2010)). Based on the central location of mRNA (Bullock et al., 1996; Moens et al., 1996; Hörsch et al., 1997) and autoradiographic GLP-1 signal (Orskov and Poulsen, 1991) and further confirmed with immunoflorescence (Tornehave et al., 2008) GLP-1R is expressed on β-cells and this expression is consistent with expression on insulinomas from rodents and humans (Göke et al., 1989; Gefel et al., 1990; Fehmann and Habener, 1991; Lankat-Buttgereit et al., 1994). There have been reports that the GLP-1R is also expressed on both α (glucagon) and δ- (somatostatin) cells in rodents (Heller and Aponte, 1995; Heller et al., 1997), however this is not supported in immunoflorescent experiments on human islets (Tornehave et al., 2008) and remains controversial. In addition to expression on islet β-cells, GLP-1R is present on the ductal exocrine cells (acinar (Xu et al., 2006; Gier et al., 2012)) an observation that may be important in relation to pancreatitis associated with the use of GLP-1 mimetics (see later).

GLP-1 has a number of physiological effects related to energy homeostasis and cardiovascular function that are mediated to some extent by the autonomic nervous system (ANS) and visceral afferent neurons. In both animals and humans GLP-1 enhances satiety (Tang-Christensen et al., 1996; Turton et al., 1996; Flint et al., 1998; Näslund et al., 1998;
Williams et al., 2009; Kanoski et al., 2011; Renner et al., 2012) and inhibition of gastric emptying (Imeryüz et al., 1997; Näslund et al., 1998; Delgado-Aros et al., 2002; Schirra et al., 2002; Nagell et al., 2006; Schirra et al., 2006; Hayes et al., 2008; Hellström et al., 2008). Inhibition of gastric emptying is regulated primarily by the ANS and satiety is modulated by both vagal afferents and direct effects on the hypothalamus with suppression of GLP-1 dependent satiety upon vagotomy (Abbott et al., 2005). There is some evidence for a GLP-1 dependent decrease in gastric acid secretion mediated by the ANS (Wettergren et al., 1994; 1997; 1998). In addition to these ANS mediated gastrointestinal effects there are also energy homeostatic effects that appear to have some ANS contribution. In humans, depending on experimental setting, there is a GLP-1 dependent (and insulin/glucagon independent) decrease in hepatic glucose production (Prigeon et al., 2003; Seghieri et al., 2013) an effect that can be replicated using intracerebroventricular (ICV) administration of GLP-1 in rodents (Sandoval et al., 2008; Burmeister et al., 2012). In mice, ICV administration of GLP-1 stimulates thermogenesis in brown adipose (Lockie et al., 2012) and lipid deposition in white adipose is decreased (Nogueiras et al., 2009). In addition, there is also evidence for GLP-1R dependent ANS regulation of the cardiovascular system in rodents (Barragán et al., 1999; Yamamoto et al., 2002; Cabou et al., 2008; Griffioen et al., 2011) but this does not appear to be the case in humans (Bharucha et al., 2008). Within the central nervous system mRNA for the GLP-1R can be detected in the thalamus, hypothalamus and brainstem in both rodents (Shughrue et al., 1996; Merchenthaler et al., 1999; Yamamoto et al., 2003) and humans (Alvarez et al., 2005) and is consistent with rodent in situ radioligand binding data (Göke et al., 1995). These central GLP-1Rs may be stimulated by circulating GLP-1, for example in privileged areas such as the subfornical organ and the area postrema which have been shown to bind peripherally administered GLP-1 (Orskov et al., 1996a), or, alternatively, may be stimulated by GLP-1 release from neurons projecting from the nucleus of the solitary tract.
There is indirect evidence in model animals for GLP-1R expression on vagal afferent nerve terminals of the hepatic portal vein (Nakabayashi et al., 1996; Nishizawa et al., 1996; Balkan and Li, 2000; Baumgartner et al., 2010) with cell bodies in the nodose ganglion (Nakagawa et al., 2004; Vahl et al., 2007). Vahl et al. (Vahl et al., 2007) use immunocytochemistry to demonstrate GLP-1R expression in hepatic portal vein nerve terminals, however it must be noted that the antibody used is no longer available from AbCam and the replacement does not recognise the GLP-1R (Panjwani et al., 2013). Similar indirect evidence suggests GLP-1R expression on enteric neurons that communicate with the vagus nerve (Washington et al., 2010).

There are conflicting reports on GLP-1/GLP-1 mimetic dependent effects on insulin sensitivity in man with some showing no effect (Orskov et al., 1996b; Vella et al., 2000; 2002) and some showing enhanced sensitivity (Egan et al., 2002; Zander et al., 2002; Meneilly et al., 2003). From the preceding discussion on the ANS, changes in liver and adipose insulin sensitivity may be influenced by the ANS (either directly or indirectly, for example via the adrenal gland e.g. (Yamamoto et al., 2002; 2003)), however there may also be direct GLP-1R mediated effects on these tissues. Whether the GLP-1R is expressed in these insulin target tissues remains contentious due to the nature of the functional experiments performed. All 3 tissue types are marked by unusual pharmacology in which the truncated form of exendin (exendin 9-39), which is normally considered an antagonist, as well as the pro-GLP-1 peptide GLP-1(1-36NH₂), which is generally considered a low affinity (e.g. (Koole et al., 2010)) both act as potent agonists in regulation of glucose uptake and metabolism in these cell types (Villanueva-Peñacarrillo et al., 1995; Wang et al., 1997; Montrose-Rafizadeh et al., 1997b; Luque et al., 2002; González et al., 2005). This has led a number of researchers to propose that a second GLP-1 receptor exists. The characterisation of exendin 9-39 and pro-GLP-1 pharmacologies as low affinity and antagonistic ligands is based
on observations in transfected cell lines. The downstream pathways linking GLP-1R to glucose uptake and metabolism are not established in these cell types. It is therefore possible that these peptides have alternative, GLP-1R dependent, pharmacologies depending on cell background, particularly given the widespread heterodimerisation of family B GPCRs (Harikumar et al., 2008).

Beyond the above tissues, which are obvious physiological targets for GLP-1, GLP-1R expression has been reported on kidney (Korner et al., 2007; Schlatter et al., 2007) and lung (Kanse et al., 1988; Richter et al., 1990; 1993; Korner et al., 2007), although expression in the human lung is restricted to small vessels (Korner et al., 2007).

**Molecular mechanisms underlying GLP-1R physiology**

GLP-1 has multiple physiological effects that include potentiation of glucose dependent insulin secretion, upregulation of insulin biosynthesis, increasing β-cell mass, suppression of glucagon secretion, delaying gastric emptying, reducing appetite, improved glucose disposal and insulin sensitivity in adipose, muscle and liver as well as improving cardiovascular function and cardiovascular protective effects. In addition GLP-1 has been reported to have anabolic effects on bone and effects on learning and memory that are beyond the scope of this review (During et al., 2003; Yamada et al., 2008).

The best studied molecular pathways underlying this physiology are those in the pancreas, in particular the β-cells (figure 1). GLP-1, acting via the GLP-1R, causes acute potentiation of GSIS, regulated primarily by signalling through the stimulatory Gα (Gαs) subunit to activate adenylate cyclase and increase intracellular cAMP (Drucker et al., 1987; Holst et al., 1987; Mojsov et al., 1987). Acute GSIS in pancreatic β-cells occurs primarily via changes in the energy balance within the cell (ATP/ADP), dependent on glucose catabolism.
Glucose transporters are not rate limiting for glucose catabolism in β-cells, rather glucokinase provides the rate limiting step and is therefore considered the glucose sensor having high cooperativity for glucose binding and an enzymatic inflection point of ~4mM. Increases in plasma glucose above this inflection point results in increased rates of glucose catabolism via the glycolytic and oxidative phosphorylation pathways in cytoplasm and mitochondria respectively. The increase in ATP/ADP ratio acts on ATP sensitive potassium channels (K_{ATP}). Binding of ATP to the pore forming subunits (K_{ir6.2})(Gribble et al., 1998) and release of ADP from the regulatory subunits (SUR1)(Gribble et al., 1997) result in channel closure leading to membrane depolarisation stimulating the opening of voltage dependent calcium channels (VDCCs) and insulin exocytosis (for reviews on GSIS see (Matschinsky et al., 1993; 1998; Schuit et al., 2001)). In vitro evidence from both rodent and human pancreatic islets demonstrates that GLP-1R-mediated increases in intracellular second messenger cAMP potentiates GSIS (reviewed in (Gromada et al., 1998)). Increases in intracellular cAMP act on the K_{ATP} channel to increase its sensitivity to ATP (i.e. left shift the ATP curve). This occurs via 2 intermediates, protein kinase A (PKA) and the guanine nucleotide exchange factor Epac2 (Ozaki et al., 2000; Kang et al., 2001; Eliasson et al., 2003). Both SUR1 and K_{ir6.2} subunits contain consensus PKA phosphorylation sites and GLP-1R dependent activation of PKA leads to phosphorylation of SUR1 subunits lowering their affinity for ADP (Light, 2002), however PKA activation also changes the dynamics of exocytosis (Eliasson et al., 2003; Hatakeyama et al., 2006; 2007) by phosphorylating snapin which acts to regulate vesicle assembly along with synaptosomal-associated protein 25 and Epac2 (Song et al., 2011). Similarly, cAMP dependent activation of Epac2 in β-cells increases the sensitivity of the K_{ATP} channel to ATP (Kang et al., 2008) and also the dynamics of vesicle priming and fusion (Hatakeyama et al., 2007; Shibasaki et al., 2007; Kang et al., 2008; Dzhura et al., 2011) thus potentiating GSIS. Epac2 activation probably converges with PKA dependent
activation of snapin (above) but additionally the activation of the small GTPase, Rap1 (Shibasaki et al., 2007) and its target phospholipase C-ε (Dzhura et al., 2011) may independently potentiate vesicle priming. These GLP-1R dependent effects to directly potentiate GSIS are Gαs/cAMP dependent, however data from the INS-1 insulinoma using supraphysiological GLP-1 stimulation in combination with β-arrestin 1 knockdown demonstrate a proportion of the cAMP/GSIS response to be β-arrestin 1 dependent (Sonoda et al., 2008). Based on the observed clinical differences in effects of GLP-1 mimetics (see later) a better understanding of the requirement for this β-arrestin 1 dependent cAMP/GSIS response may be required for the development of small molecule drugs. In addition to post-glucokinase effects, GLP-1R mediated activation of Epac2 acting in a Rim2/Rab3A dependent manner (in rodent cell lines and islets) acutely decreases the Kₘ (but not Vₘₐₓ) of glucokinase (Park et al., 2012), thus providing an additional sensitisation to GSIS, albeit at supraphysiological GLP-1 concentrations.

In addition to acute potentiation of GSIS, GLP-1R activation improves medium and long term insulin secretion via a number of mechanisms. In insulinoma cell lines GLP-1R activation leads to increases in insulin expression. This effect is due to both stabilisation of insulin mRNA and increases in insulin transcription (Fehmann and Habener, 1992; Wang et al., 1995). GLP-1R activation in insulinoma cell lines activates cAMP response element binding protein (CREB) and NFAT in both PKA dependent and independent manners to activate insulin transcription (Skoglund et al., 2000; Kemp and Habener, 2001; Chepurny, 2002; Lawrence et al., 2002). The molecular pathways that connect GLP-1R to insulin transcription are however unclear, as it has been shown that GLP-1R activation can induce insulin expression independent of cAMP, PKA and Epac2. Insulin secretion is also dependent on β-cell mass and GLP-1R activation is associated with trophic and protective effects including proliferation, anti-apoptosis and stimulation of islet neogenesis. In insulinoma cell
lines activation of GLP-1R generates cell autonomous signaling via β-arrestin 1 to inhibit the activity of pro-apoptotic protein Bad (Quoyer et al., 2010) and paracrine pro-proliferative effects via post-transcriptional upregulation of epidermal growth factor receptor (EGF-R1) (Buteau et al., 2003; Cornu et al., 2010). Isolated human islets are also protected from apoptosis by GLP-1 (Farilla et al., 2003), however this may be compensatory due to loss of tonic stimulation of cAMP (compare (Xie et al., 2007) and (Preitner et al., 2004)). In glucose intolerant rats, GLP-1 increases β-cell mass correlating with induction of expression of the β-cell transcriptional regulator pancreatic-duodenum homeobox-1 (PDX-1) (Perfetti et al., 2000). GLP-1R dependent activation of PDX-1 is also seen in model insulinomas and is dependent on a cAMP/PKA pathway (Wang et al., 2001) and induction of PDX-1 expression is likely to contribute directly to islet neogenesis (Hui et al., 2001; Bulotta et al., 2002). In addition to these direct effects, vagal innervation of the pancreas is well documented to regulate β-cell mass (e.g. (Lausier et al., 2010; Llewellyn-Smith and Verberne)) and GLP-1R activation stimulates pancreas projecting vagal neurons (Wan et al., 2007a; 2007b). Beyond GLP-1R mediated effects on the endocrine pancreas, rodent models show significant adverse effects in exocrine pancreas. In a 10 week trial in rats, exendin treatment resulted in significant acinar cell death and inflammation (Nachnani et al., 2010) and in a mouse model of pancreatic intraepithelia neoplasia, exendin treatment significantly accelerated metaplasia and lesion formation (Gier et al., 2012). While there is no mechanistic data to explain these exocrine pancreatic effects, recent meta analysis on the incidence of pancreatitis in T2DM patients shows a doubling in relative risk for those using GLP-1 therapies (Singh et al., 2013). The pleiotropic effects on β-cell function and mass are clinically very important as preservation of pancreatic function is one of the key advantages to GLP-1 based therapies. It is also imperative that the nature of GLP-1 pathways that form the molecular basis for
pancreatitis be established. These pathways may be via changes in pancreatic morphology due to accelerated β-cell neogenesis or via direct effects on acinar cells that express GLP-1R.

The ability of GLP-1 to suppress glucagon secretion from pancreatic α-cells is regarded as important for its glucoregulatory effects. Under conditions of hyperinsulinemic-euglycemic clamp, GLP-1 does not inhibit glucagon secretion in humans (Meneilly et al., 2003). This suggests that GLP-1 dependent regulation of glucagon secretion is not mediated by direct effects on pancreatic α-cells but rather is mediated by a combination of pancreatic paracrine (Franklin et al., 2005) and ANS signalling (Llewellyn-Smith and Verberne).

The ANS effects of GLP-1 are critical for the success of GLP-1 mimetics as treatments for T2DM. The effect of GLP-1 mimetics on post-prandial glucose excursion is largely via slowing in gastric emptying (Meier et al., 2005; Linnebjerg et al., 2008), however this effect is subject to tachyphylaxis (Nauck et al., 2011) and as a result, long acting GLP-1 analogues are less efficacious in controlling post-parandial glucose excursion (Degn et al., 2004; Buse et al., 2009). GLP-1 dependent decreases in gastric emptying appear to be mediated by the ANS (Nagell et al., 2006; Holmes et al., 2009) suggesting that the sites of GLP-1R expression responsible for gastric emptying are sites in which GLP-1R is subject to agonist mediated desensitisation. In contrast, similar weight loss is observed in T2DM patients receiving both long and short acting GLP-1 mimetics (Drucker et al., 2008; Buse et al., 2009), consistent with the gastric motility independent decrease in food intake seen in ICV treated rats (Turton et al., 1996) that is apparently not subject to GLP-1R desensitisation.

It is therefore likely that there are some sites in these pathways where GLP-1R is desensitised and some are not and this is likely to be an important consideration for the design of small molecule GLP-1R agonists. There is one site in which GLP-1R stimulation has been shown to result in neuronal depolarisation (e.g. (Wan et al., 2007a; 2007b; Holmes et al., 2009)) however there is no data that addresses the pathways immediately downstream of the GLP-
GLP-1R that cause depolarisation and there is no data that allows this site to be extrapolated to sites important for gastric emptying and satiety. An understanding of the subsets of neurons activated and the downstream pathways required for the therapeutic effects is important as there are clear differences in the anorectic effects of different GLP-1R agonists. In rodents GLP-1 and exendin drive similar reductions in food intake when administered peripherally but exendin is far more potent when administered ICV (Barrera et al., 2009); in clinical trials high molecular weight GLP-1 mimetics (e.g. Albiglutide and Dulaglutide) fail to provide weight loss benefits seen with peptide mimetics (Rosenstock et al., 2009; Grunberger et al., 2012) suggesting differential access by these ligands to central GLP-1R.

As part of its overall glucoregulatory role, GLP-1 can act to inhibit endogenous glucose production in the liver both acutely (Meneilly et al., 2003; Prigeon et al., 2003) and over the long term (Egan et al., 2002; Zander et al., 2002). Although GLP-1 binding has been demonstrated on rat hepatocyte membranes (Villanueva-Peñacarrillo et al., 1995) there is no convincing evidence that GLP-1 agonists exert a direct effect on liver metabolism, rather GLP-1 effects on hepatic function, where they occur, are likely to be centrally mediated (although this may be indirect). In human adipocytes there is one report showing GLP-1 potentiation of glucose uptake via PI3K and MAPK pathways (Sancho et al., 2007). In this report, exendin 9-39 also stimulates PI3K activity which contrasts to the traditional view of this ligand as an antagonist. In addition, the data from the mouse suggests that, rather than stimulating glucose uptake and therefore lipogenesis, GLP-1 acts to increase lipolysis. Given one of the useful clinical outcomes of GLP-1 mimetics is sustained weight loss, a better understanding of the effects on adipose physiology is required.

Our understanding of the underlying molecular basis of GLP-1’s pleiotropic effects would benefit greatly from high quality antibodies directed against GLP-1R and a GLP-1R reporter mouse so the precise location of receptors in different tissues could be fully
characterised. Better characterisation of pathways leading to translational and post-translational control of gene expression by GLP-1R activation is required. Additionally, comprehensive studies that provide pharmacological or biochemical characterisation of GLP-1R on tissues such as liver and adipose would greatly enhance our understanding of the underlying physiology.

**Challenges for incretin therapies for T2DM**

Currently there are no therapeutically approved small molecule GLP-1R agonists. As discussed above, the therapeutic usefulness of GLP-1 (peptide) is limited by its metabolic instability. Thus, therapeutic strategies for increasing active GLP-1 concentrations include DPP4 resistant GLP-1 mimetics such as exenatide and liraglutide or DPP4 inhibitors such as vildagliptin and sitagliptin which are approved as monotherapies and/or adjuvant drugs with oral antidiabetic compounds, depending on the country of approval.

Approved DPPIV inhibitors (known as gliptins) show very good selectivity for DPPIV over the structurally related enzymes DPPVIII and DPPIX (Brandt et al., 2005; Kim et al., 2005; Feng et al., 2007; Wang et al., 2012). DPPIV inhibitors produce modest changes in circulating levels of active GLP-1 (GLP-1(7-36)NH₂) levels ranging from 2-4 fold depending on the study (Ahrén et al., 2004; Herman et al., 2005; Dai et al., 2008; Henry et al., 2011). For healthy individuals this corresponds to an increase in fasted GLP-1 to ~3pM and post-prandial peak of ~18pM, compared with T2DM patients where reduced GLP-1 secretion is observed and the plasma concentrations are roughly half. Although the available data indicate that DPPIV inhibition is a promising treatment for type 2 diabetes, gliptins are clinically less effective than GLP-1 mimetics (reviewed in (Brown and Evans, 2012; Reid, 2012; Scheen, 2012)). There are also concerns that prolonged inhibition of DPPIV activity could lead to adverse side effects. Substrate cleavage by DPPIV occurs at penultimate L-
proline or L-alanine residues (Kenny et al., 1976). Some known and putative in vivo substrates of DPPIV include substance P (Heymann and Mentlein, 1978; Kato et al., 1978), neuropeptide Y and peptide YY (Mentlein et al., 1993a), endomorphin-1 (Bird et al., 2001), pituitary adenylate cyclase activating peptide 38 (PACAP 38) (Lambeir et al., 2001); GLP-1 and GIP (Mentlein et al., 1993b), GLP-2 (Drucker et al., 1997) and various chemokines such as CCL5 (regulated on activation, normal T-cell expressed and secreted, RANTES) and CCL11 (eotaxin) (Oravecz et al., 1997), CCL22 (macrophage derived chemokine, MDC) (Proost et al., 1999), CCL3L1 (LD78β) (Proost et al., 2000) and CXCL12 (stromal-cell-derived factor 1, SDF-1) (Proost et al., 1998). Thus, inhibition of DPPIV could potentially extend the circulating half-lives of these biologically active peptides which might conceivably affect/modulate vasoreactivity, nociception, energy homeostasis (food intake, lipid metabolism, thermogenesis, and glucose control), proliferation, angiogenesis, immune response, behavioural stress response, gastrointestinal motility and growth.

Currently there are a number of GLP-1 analogues that have been approved or are in clinical trials for treatment of T2DM (figure 2). The main drawback of all GLP-1 analogues is the need for parenteral administration. There has therefore been substantial effort to extend the half-life of these mimetics. Two synthetic analogues of exendin-4, exenatide and lixisenatide, were isolated from the saliva of the Gila monster (Heloderma suspectum). These 2 analogues are naturally resistant to DPPIV; exenatide and lixisenatide, which has a 6 amino acid polylysine extension at the C-terminus, have circulating half lives of around 4 hours but have been formulated for once a day injection. Liraglutide is based on the mammalian GLP-1 sequence with a glutamate and 16 carbon fatty acid conjugated to the ε-amino group of lysine26 and a substitution of lysine34 with arginine. This modification increases albumin binding to 99%, thus protecting liraglutide from DPPIV degradation and giving it an 11-13 hour half life. Taspoglutide is also based on mammalian GLP-1 with substitution of Ala8 and
Ala35 with methylated derivatives, thus protecting the peptide from DPPIV and giving a half-life of 10 hours. Albiglutide uses 2 molecules of gly8 substituted GLP-1 sequences covalently coupled to human albumin and has a half-life of 6-8 days. Dulaglutide uses 2 molecules of a position 8 valine substituted GLP-1 sequence fused via a linker to human IgG4-Fc domain and has a half-life of 4 days. Lastly exenatide-LAR is a microsphere formulated extended release formulation of exendin that is suitable for once a week injection. These GLP-1 analogues are very selective for GLP-1R activation and do not suffer from the potential off-target affects of DPPIV inhibitors. Clinically, all GLP-1 mimetics are better at lowering fasting & post prandial plasma glucose, glycosylated haemoglobin levels and weight compared with DPPIV inhibitors (Arnolds et al., 2010; Pratley et al., 2010; Berg et al., 2011; Pratley et al., 2011). In spite of these positive attributes there are differences in the clinical outcomes from trials using these different analogues that point to differential GLP-1R activation or signal bias by these ligands as well as safety concerns. In spite of the relatively minor changes in sequence for taspoglutide compared with mammalian GLP-1, this drug has now been withdrawn from clinical trials due to unacceptably high incidence of nausea and vomiting as well as systemic and injection site allergic reactions (Rosenstock et al., 2013). It is unclear why this mimetic displays such a different emetic effect compared with liraglutide which has similar pharmacokinetics, however we would speculate that this may be due to differences in the mechanism of GLP-1R activation or access. Additionally, GLP-1 desensitisation may play an important role in the tolerability of GLP-1 mimetics. All GLP-1 mimetics have nausea and vomiting as side effects, however these side effects generally subside over time. It is therefore possible that the clinical difference seen with Taspoglutide is due to failure of reeptor desensitisation. Although exenatide-LAR, albiglutide and dulaglutide offer the benefit of once weekly injection, these long acting analogues do not offer the same level of weight reduction seen with the short acting analogues and this appears
to be due to their reduced ability to promote satiety. The large conjugates, albiglutide and dulaglutide also appear to be poor potentiators of GLP-1R anorectic effects and this is probably due to the inability of these large molecules to access areas of the CNS important for this effect. Alternatively, all long acting mimetics may be less effective at promoting satiety due to them causing receptor desensitisation in parts of the ANS responsible for satiety. If this is the case a thorough understanding of the different neuronal types and the mechanisms for GLP-1R desensitisation in these neurons will be necessary. Lastly, although evidence has not been published for albiglutide and dulaglutide, both DPPIV inhibitors and GLP-1 mimetics double the risk of pancreatitis, a condition associated with serious morbidity. This effect is almost certainly due to GLP-1R activation on acinar cells of the exocrine pancreas leading to their apoptosis and metaplasia and causing associated inflammation. In pancreatic β-cells, GLP-1R activation has anti-apoptotic and pro-proliferative effects, whereas in acinar ductal cells it appears to be pro-apoptotic and drive metaplastic differentiation. In a subset of neurons responsible for GLP-1 mediated gastric emptying, sustained activation does not appear to be susceptible to tachyphylaxis whereas the neurons responsible for appetite suppression are. It may well be possible to generate small molecule ligands for the GLP-1R that display a suitable bias profile so as to bypass the pancreatic intraepithelial neoplasia while improving the weight loss profile through lack of receptor desensitisation in the CNS. For these challenges to be met, a better understanding or key aspects of GLP-1R physiology and underlying molecular signalling is required.

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Conflict of interest

None declared.

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Processing by CD26/dipeptidyl-peptidase IV reduces the chemotactic and anti-HIV-1 activity

Truncation of macrophage-derived chemokine by CD26/ dipeptidyl-peptidase IV beyond its
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Table 1
Comparison of therapies for type 2 diabetes in terms of therapeutic outcome and adverse events (TZDs = Thiazolidinedione; HbA1c = glycated haemaglobin).

<table>
<thead>
<tr>
<th></th>
<th>Sulfonylureas</th>
<th>Metformin</th>
<th>TZDs</th>
<th>DPP4 inhibitors</th>
<th>GLP-1 mimetics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Short acting</td>
</tr>
<tr>
<td>Insulin secretion</td>
<td>Increased</td>
<td>No effect</td>
<td>No effect</td>
<td>Glucose dependent increase</td>
<td>Glucose dependent increase</td>
</tr>
<tr>
<td>β-cell glucose sensitivity</td>
<td>No effect</td>
<td>Increased</td>
<td>No effect</td>
<td>Increased</td>
<td>Increased</td>
</tr>
<tr>
<td>Target tissue insulin sensitivity</td>
<td>No effect</td>
<td>Increased</td>
<td>Increased</td>
<td>Unclear</td>
<td>Unclear</td>
</tr>
<tr>
<td>β-cell mass</td>
<td>No protection from loss</td>
<td>No protection from loss</td>
<td>No protection from loss</td>
<td>Unclear</td>
<td>Probably increased</td>
</tr>
<tr>
<td>HbA1c</td>
<td>Reduced</td>
<td>Reduced</td>
<td>Reduced</td>
<td>Reduced</td>
<td>Reduced</td>
</tr>
<tr>
<td>Satiety</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect</td>
<td>Increased</td>
<td>Increased</td>
</tr>
<tr>
<td>Gastric emptying</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect</td>
<td>Reduced</td>
<td>Reduced</td>
</tr>
<tr>
<td>Weight</td>
<td>Increase</td>
<td>neutral</td>
<td>Increase</td>
<td>neutral</td>
<td>Sustained loss</td>
</tr>
<tr>
<td>Long term insulin dependency</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Unlikely</td>
<td>Unlikely</td>
</tr>
<tr>
<td>Pancreatitis</td>
<td>Not reported</td>
<td>Not reported</td>
<td>Not reported</td>
<td>Increased risk</td>
<td>Increased risk</td>
</tr>
<tr>
<td>Adverse GIT effects</td>
<td>Broad spectrum GIT AEs</td>
<td>Mostly absent</td>
<td>Mostly absent</td>
<td>Some nausea</td>
<td>Some nausea</td>
</tr>
</tbody>
</table>
| hypoglycemia         | At risk       | Low risk   | none | none | none | none

**Figure legends**

Figure 1. Cartoon depicting the major pathway by which a pancreatic β-cell secretes insulin in response to increases in plasma glucose. The various pathways that originate from GLP-1R activation and converge to potentiate glucose stimulated insulin secretion are depicted. See text for a full description.
Figure 2. A schematic of the various GLP-1 mimetics discussed in the text. Differences in sequence with respect to native GLP-1 are shown in red. Albiglutide is a genetic fusion of a GLP-1 concatamer to human albumin (blue) and Dulaglutide is a genetic fusion of GLP-1 via a linker (red) to the Fc domain of human IgG4 (blue).
pro-GLP-1
HDEFERHAEGTFTSDVSSYLEQAAKEFIAWLVKGRG

GLP-1 (7-36)NH$_2$
HAEFTSDVSSYLEQAAKEFIAWLVGR-NH$_2$

Exenatide
HGEFTSDLSKQMEAAVRLFiewLKNGPPSSGAPPS

Lixisenatide
HGEFTSDLSKQMEAAVRLFiewLKNGPPSSGAPPSKKKKK

Liraglutide
HAEFTSDVSSYLEQAAKEFIAWLVRGR-NH$_2$

Taspoglutide
HAEFTSDVSSYLEQAAKEFIW$^*$AGR-NH$_2$

Albiglutide
HGEFTSDVSSYLEQAAKEFIAWLVGRGHGEFTSDVSSYLEQAAKEFIAWLVKRG

Dulaglutide
HVEGTFTSDVSSYLEQAAKEFIAWLVKRG
HVEGTFTSDVSSYLEQAAKEFIAWLVKRG

Figure2.jpg