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Review

Landmark studies on the glucagon subfamily of GPCRs: from small molecule modulators to a crystal structure

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The glucagon subfamily of class B G-protein-coupled receptors (GPCRs) has been proposed to be a crucial drug target for the treatment of type 2 diabetes. The challenges associated with determining the crystal structures of class B GPCRs relate to their large amino termini and the lack of available small molecule ligands to stabilize the receptor proteins. Following our discovery of non-peptidic agonists for glucagon-like peptide-1 receptor (GLP-1R) that have therapeutic effects, we initiated collaborative efforts in structural biology and recently solved the three-dimensional (3D) structure of the human glucagon receptor (GCGR) 7-transmembrane domain, providing in-depth information about the underlying signaling mechanisms. In this review, some key milestones in this endeavor are highlighted, including discoveries of small molecule ligands, their roles in receptor crystallization, conformational changes in transmembrane domains (TMDs) upon activation and structure-activity relationship analyses.

Keywords: class B GPCR; GLP-1R; GCGR; small molecule modulators; crystal structure; type 2 diabetes mellitus

Introduction

The prevalence of diabetes is increasing worldwide. Most cases are type 2 diabetes mellitus (T2DM), which parallels a marked lifestyle transition and a worldwide epidemic of obesity in both developed and developing countries. Last year, the American Diabetes Association announced that 29.1 million Americans, representing 9.3% of the population, had diabetes in 2012, whereas in China it affected 113.9 million people, or 11.6% of the Chinese adult population in 2010[1, 2]. These large national surveys indicate that diabetes has become a major public health problem, as well as a leading cause for disability and mortality in modern societies. The cardinal symptoms of T2DM are hyperglycemia and subsequent complications, which result in the main therapeutic strategies relying heavily on the management of hyperglycemia, including the use of metformin, sulfonylurea, thiazolidinedione, insulin, and inhibitors or modulators of sodium glucose cotransporter 2 (SGLT2), dipeptidyl peptidase 4 (DPP-4) and glucagon-like peptide-1 receptor (GLP-1R) agonists. For these indications, target-based drug discovery has been a focal point

of research for decades[3].

G protein-coupled receptors (GPCRs), of which there are more than 800, have heptahelical transmembrane domains (TMDs) and represent the richest source of therapeutic targets for the pharmaceutical industry[4]. They mediate cellular responses to external stimuli via their extracellular face; they transmit this extracellular signal and elicit various activities in the cell through structural transitions of their intracellular face[5]. This signal transduction is realized by G proteins, which are coupled to second messengers, such as cAMP, inositol 1,4,5-trisphosphate (IP3), and Ca^{2+}, and perform vital functions in vision, olfactory perception, metabolism, the endocrine system, neuromuscular regulation and the central nervous system (CNS)[6]. The GPCR family is divided into five main subfamilies—rhodopsin (class A), secretin (class B), glutamate (class C), frizzled (class F) and adhesion (others)[7]—endowing the cell with remarkably versatile functions. Understanding the three-dimensional (3D) structures of GPCRs will help elucidate their functionalities. However, the highly conserved hydrophobic core of GPCRs and its inherent flexibility cause enormous difficulties for protein purification and crystallization. To date, the crystal structures of 23 class A GPCRs have been determined[8-10], increasing our knowledge of the molecular mechanisms of ligand binding and receptor

activation and facilitating structure-based drug design.

In addition to a seven-transmembrane fold similar to that in class A GPCRs, class B receptors feature a large extracellular N-terminal region, which is responsible for binding to peptide ligands of 30–40 amino acid residues. The extracellular domains (ECDs) of class B GPCRs have been structurally elucidated in complex with their cognate ligands, revealing some common features of peptide hormone recognition[11]; however, it has been difficult to obtain crystal structures of full-length class B GPCRs, owing to considerable technical hurdles in receptor production, purification, and protein stability and homogeneity (including difficulties in finding optimal protein constructs, buffer conditions, lipids, detergents and high-affinity small molecule modulators). Structures of the TMDs of class B GPCRs were not available until the recent publication of the structures of human glucagon receptor (GCGR) and human corticotropin-releasing factor receptor 1 (CRF₁R)[12, 13]. In this review, we focus on the glucagon subfamily of class B GPCRs and summarize the key milestones in studies on the structure and function of GCGR and GLP-1R, including discoveries of small molecule ligands, their roles in the receptors' crystallization, the conformational changes in TMDs upon activation and structure-activity relationship (SAR) analyses.

Class B GPCRs

The class B GPCRs are a family of receptors with 15 members that interact with a variety of therapeutically relevant peptide hormones, including secretin, glucagon, GLPs, vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating peptide (PACAP), growth hormone-releasing factor (GRF), gastric inhibitory polypeptide or glucose-dependent insulinotropic polypeptide (GIP), corticotropin-releasing factor (CRF), parathyroid hormone (PTH) and calcitonin[11, 14-20]. When developing therapeutics, a major drawback of these endogenous peptides is their very short half-lives, which result from rapid degradation[21]. Therefore, the development of stable non-peptidic agonists for class B GPCRs is an important mission. This is in marked contrast to class A GPCRs, for which antagonists rather than agonists have been actively pursued[8]. Despite extensive efforts made by the pharmaceutical industry, there has been little success in developing drug-like nonpeptidic ligands for class B GPCRs.

Each class B GPCR consists of a large N-terminal disulfide-linked ECD, and the crystal structures of ECDs bound to several cognate peptide ligands have revealed a consensus mechanism for hormone binding[11]. ECD binding invokes *a*-helix formation by the hormone, which in turn leads to receptor activation. Currently, 9 of the 15 class B GPCR ECD structures have been determined, all showing that the C-terminal *a*-helix of the ligand binds to the β-sheet of the ECD, with the exception of PACAP, which displayed a bend at the Ala18 residue[22-32]. These studies provide important information about the structural basis of ligand recognition and selectivity.

GCGR and GLP-1R

The glucagon subfamily of class B GPCRs includes GCGR, GLP-1R, glucagon-like peptide-2 receptor (GLP-2R) and GIP receptor (GIPR), whose natural ligands share a high degree of sequence homology at their amino termini. This subfamily of receptors plays important roles in regulating glucose homeostasis, with the ligand glucagon acting to maintain blood glucose levels via the conversion of glycogen to glucose and incretins reducing blood glucose levels primarily through stimulating the release of insulin. These receptors are of considerable interest as targets in the treatment of metabolic disorders. In particular, a number of GLP-1R peptidic agonists, which are resistant to enzymatic breakdown by dipeptidyl peptidase-4 (DPP-4), have been shown to be highly effective in the treatment of T2DM and obesity, not only lowering blood glucose but also eliciting weight loss[33].

GCGR is a 485 amino acid protein with a high affinity for glucagon binding. It has been identified in the liver, kidney, intestinal smooth muscle, brain and adipose tissue[34]. GCGR is also expressed in pancreatic islet β-cells, which are involved in the regulation of insulin secretion[35]. In addition to its link to intra-hepatic glucose metabolism, GCGR expression is positively regulated by glucose and negatively regulated by glucagon and agents that increase intracellular cAMP[36]. Experiments using GCGR and GLP-1R antagonists suggest that glucagon activates β-cell signaling pathways via both receptors[37]. Disruption of GCGR activity in *Gcgr*⁻/⁻ mice led to a significant increase in total pancreatic weight, marked islet α-cell hyperplasia and an elevation of circulating glucagon and GLP-1 levels, accompanied by multiple defects in the development of islet cell phenotypes[38]. These results give important insights into the role of GCGR in pancreatic islet β-cells beyond the control of hepatic glucose production.

Human GLP-1R consists of 463 amino acids and exhibits approximately 47% sequence identity with GCGR. It has been localized to multiple tissues and cell types in the pancreas, central and peripheral nervous systems, gastrointestinal tract, cardiovascular system, kidney and lung[39]. Its pancreatic localization is primarily in β-cells, and the natural peptide ligand is GLP-1, whose importance for regulating postprandial blood glucose levels has been known for decades. Likewise, impairment of incretin function in T2DM is widely accepted and has led to the development of stable GLP-1 analogues for T2DM treatment[40, 41]. In pancreatic β-cells, GLP-1 exerts its insulinotropic action via GLP-1R, which leads to the initiation of numerous intracellular signaling pathways, such as the Gαs-mediated cAMP pathway, calcium mobilization and MAP kinase activation[42]. A major goal for the treatment of T2DM has been the development of orally active, non-peptidic GLP-1R agonists[43, 44]. Although lead compounds have been reported in the literature, progress has been slow to date.

Other subfamily members

The human GLP-2R gene is localized on chromosome 17p13.3 and shares approximately 50% sequence identity with GLP-1R[45]. It is expressed predominantly in the gastrointestinal tract, and its biological actions have been implicated in gut injury, short bowel syndrome and adaptive mucosal responses

to re-feeding[45]. Similar to GLP-1R, GLP-2R also plays important roles in glucose intolerance and hepatic insulin resistance[46]. The elimination of GLP-2R signaling in obese mice impaired the normal islet adaptive responses required to maintain glucose homeostasis[46]. To date, only one small molecule modulator, which was determined to be of ago-allosteric nature, has been reported for this receptor[47]. GIPR is widely expressed in the pancreas, stomach, small intestine, adipose tissue, adrenal cortex, pituitary gland, heart, testis, endothelial cells, bone, trachea, spleen, thymus, lung, kidney, thyroid and several regions of the CNS. It is thought that this hormone is involved in physiological processes such as bone quality[48], memory formation and the control of appetite[49, 50], as well as the pathogenesis of obesity and insulin resistance[51]. While GLP-2R and GIPR might be crucial for understanding obesity and diabetes, little research has been conducted in terms of chemical and structural biology. Therefore, this review will focus on GCGR and GLP-1R.

Small molecule GLP-1R modulators

Compounds **1** (T-0632)[52] and **2** (catechin, see Table 1)[53] were reported to be GLP-1R antagonists. Compound **1**, originally identified as a cholecystokinin receptor 1 (CCK1) antagonist, is a non-competitive antagonist of human GLP-1R with low micromolar potency and is presumably capable of binding to the ECD (a large independently folded globular ectodomain at the N-termini of class B GPCRs) of GLP-1R[52]. However, its low affinity for GLP-1R combined with its sub-nanomolar CCK1 antagonist activity makes it not a useful molecular tool. Compound **2** is a polyphenolic natural product that functionally acts as a selective negative allosteric modulator of GLP-1R[53]. It displays signaling bias in a peptide-specific manner. In calcium mobilization assays, catechin had no effect on agonist-mediated signaling; however, in cAMP accumulation assays, catechin acted as a negative modulator of both GLP-1$_{(7-36)}$NH$_2$ and GLP-1$_{(1-36)}$NH$_2$. Furthermore, the potency of GLP-1$_{(7-37)}$ in stimulating cAMP production was also significantly reduced (8-fold) in the presence of catechin, whereas cAMP responses to GLP-1$_{(1-37)}$, exendin-4 and oxyntomodulin were unaltered.

Compounds **3-8** were reported to be GLP-1R agonists. Quinoxaline derivatives, represented by compounds **3** and **4**, were discovered by Novo Nordisk[54]. An initial screening of 500 000 small molecules in a competition-binding assay did not find any useful hits. A subsequent functional screening of 250 000 chemically diverse compounds and follow-up SAR studies led to the discovery of these quinoxaline derivatives. Compound **4** was characterized as a selective ago-allosteric GLP-1R agonist as its activity could not be blocked by exendin$_{9-39}$. It increased the binding affinity of both GLP-1 and oxyntomodulin for GLP-1R and promoted glucose-dependent insulin secretion in wild-type mouse islets but not in GLP-1R knockout mice.

Then, a series of pyrimidine derivatives exemplified by compound **5** (BETP) was discovered, which have key structural differences from the previously reported quinoxalines. The pyrimidines require both trifluoromethyl and sulfonyl groups and an electron deficient heterocycle for strong activity, whereas the quinoxalines require one of the functional groups and an electron-rich ring system. Compound **5** demonstrated dose-dependent activities in both cAMP accumulation and insulin secretion assays. Similar to the quinoxalines, BETP exhibited agonist and positive allosteric activities for GLP-1R, and its agonist actions could not be blocked by exendin$_{9-39}$[55]. Compound **6** is a GLP-1R agonist with an oxadiazoanthracene skeleton[56, 57]. It showed a nanomolar potency for GLP-1R in cAMP assays, and it stimulated glucose-dependent insulin secretion by rodent islets and improved glucose excursion in an oral glucose tolerance test[58, 59].
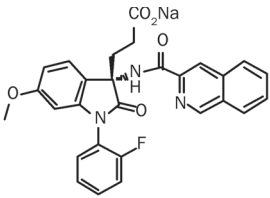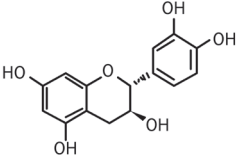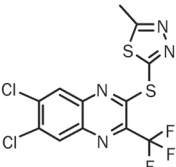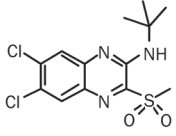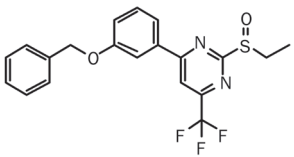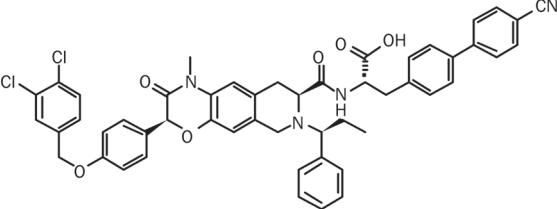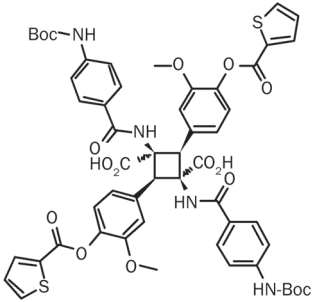
Full GLP-1R agonists, represented by compound **7** (Boc5) and its cyclopentyl derivative (S4P, **8**), were reported by our group in 2007[43]. They are a new class of cyclobutanes with four chiral carbons. The X-ray crystal structure of compound **7** indicates that it adopts an *anti* head-to-tail 1,2-*cis*-2,3-*cis*-3,4-*cis* configuration in which the four side chains of the core point in the same direction, similar to the four legs on a stool[60]. The bioactivities of compound **7** and its three steric isomers differ significantly in both GLP-1R binding and MRE/CRE-driven reporter gene assays, with compound **7**, the most potent GLP-1R agonist, exhibiting as high as 96.7% efficacy relative to the native peptide, GLP-1. It is also a selective GLP-1R agonist because it does not activate reporter gene activity of other related GPCRs, such as GLP-2R, GCGR and GIPR[43, 61]. Following chronic intraperitoneal administration to diabetic *db/db* mice, compound **7** lowered HbA1c, reduced food intake, decreased body weight and enhanced insulin secretion[61, 62]. These therapeutic effects were also reproduced by a derivative of compound **7** called WB4-24 (an isobutyryl derivative)[60]. However, the poor oral bioavailability and undesirable structural characteristics of these compounds make them undruggable (this class of molecules violates all of the Lipinsky and Veber rules)[63].
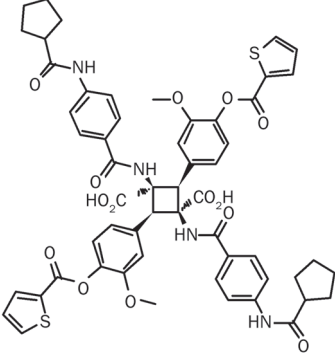
Small molecule GCGR modulators

A series of β-alanines, represented by compounds **9-11** (Table 2), were reported to be ligands for both GLP-1R and GCGR. Four libraries containing 1056 compounds were designed to avoid the potential toxicity of the 2-chloropyridyl group in compound **9**[64]. The results were as follows: 1) the 2-chloropyridyl group could be replaced by other aromatic groups such as benzothiophene; 2) the large biphenylpropyl group could be replaced by smaller sized and lower lipophilicity groups such as 4-*tert*-butylbenzyl; 3) the central amide could be replaced by urea, keeping the important binding moieties together in a suitable geometry for the binding of the ligand to the receptor; and 4) compounds with either small electron-withdrawing or larger lipophilic groups in the para position of the distal phenyl moiety increased the potency.

This led to the discovery of compounds **10** and **11**, both of which are orally available in dogs and capable of decreasing blood glucose levels in diabetic animals, with **11** having better pharmacokinetic properties. Their selectivity for GLP-1R

Table 1. Small molecule modulators for glucagon-like peptide-1 receptors.

No	Chemical structure	Bioactivity	Reference
1 (T0632)		Non-competitive antagonist	[52]
2 (Catechin)		Selective and negative allosteric modulator	[53]
3		Agonist	[54]
4		Selective ago-allosteric agonist	[54]
5 (BETP)		Positive allosteric agonist	[55]
6		Agonist	[56-59]
7 (Boc5)		Full agonist	[43, 60-62]

(To be continued)

No	Chemical structure	Bioactivity	Reference
8 (S4P)		Agonist	[43, 60-62]

and GCGR was also studied; both compounds displayed weak binding affinities for GLP-1R (IC$_{50}$=1 μmol/L for **10** and 4.9 μmol/L for **11**) with **11** having 180-fold selectivity for GCGR over GLP-1R.

Cascieri *et al* developed a series of triarylimidazole and triaryl-pyrrole derivatives, represented by compound **12** (L-168,049), with selective binding affinity for GCGR. Compound **12** inhibited the binding of radiolabeled glucagon to GCGR with an IC$_{50}$ value of 3.7 nmol/L, without any effects on the binding of labeled GLP-1 to GLP-1R at concentrations up to 10 μmol/L[65]. Acylated aminothiophene-3-nitrile derivatives, represented by compound **13**, were reported by Duffy *et al* to be GCGR antagonists. Compound **13** displayed moderate binding (IC$_{50}$=181 nmol/L) and cAMP accumulation activities (IC$_{50}$=129 nmol/L). Further structural modifications led to the development of compound **14**, which improved potencies for both receptor binding (IC$_{50}$=89 nmol/L) and cAMP accumulation (IC$_{50}$=34 nmol/L)[66].

As a continuation, Lee *et al* incorporated a cyclic core (pyrrolidine or cyclopentane) to restrain the conformation and developed compound **15**[67], which was disclosed as a novel GCGR antagonist. They replaced the central phenyl ring with a pyrimidine to obtain two enantiomers, (+) **16** and (-) **16**. Both of these compounds were resistant to oxidative metabolism in human, rat and dog liver microsomes *in vitro*. *In vivo* pharmacokinetic studies on (+) **16** and (-) **16** were also conducted in Wistar-Han rats and in dogs. However, a high plasma clearance, especially for (+) **16** (Cl=56.7 mL·min^{-1}·kg^{-1}), was observed in rats. Re-examination in bile duct-cannulated rats revealed that biliary excretion was a major route for drug elimination, even though 40%-45% of the dosed parent compounds was unchanged. Interestingly, a significant difference in clearance was observed in dogs [25.8 mL·min^{-1}·kg^{-1} for (+) **16** and 2.7 mL·min^{-1}·kg^{-1} for (-) **16**], which may be caused by an enantio-specific interaction with the biliary efflux transporter(s) in dogs[68].

Further structural modification was carried out by introduc-

ing spiroimidazolone to obtain compound **17** and its bioisostere compound **18** (a tetrazole derivative, SCH 900822), and by introducing a pyrazole to obtain compound **19**. Compound **18** exhibited better selectivity, and oral dosing with **18** lowered 4-h fasting and 24-h non-fasting glucose levels in diet-induced obese (DIO) mice and 8-h fasting glucose levels in streptozotocin-induced type 2 diabetic mice[69]. Compound **19** (MK-0893) was reported to be a reversible and competitive GCGR antagonist that does not cross react with other class B GPCRs, including GIPR, PAC1, GLP-1R, VPAC1 and VPAC2. In insulin-resistant *ob/ob* mice, compound **19** lowered blood glucose levels by up to 39% with acute oral administration, whereas in DIO mice receiving chronic oral treatment, compound **19** decreased blood glucose to the levels observed in lean counterparts and stabilized these levels for the duration of the study[70].
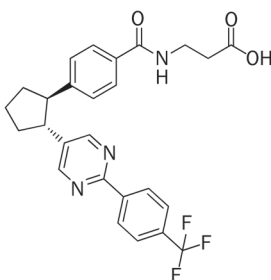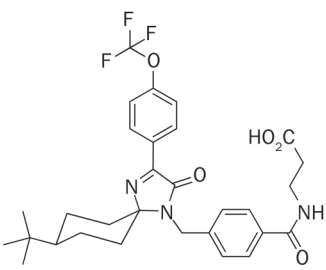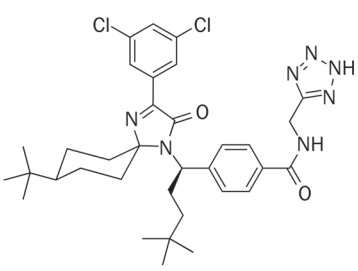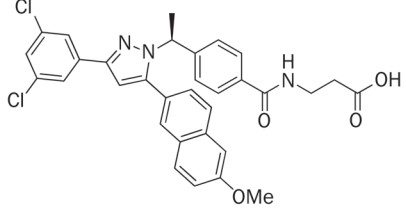
Structure-activity relationship studies

Small molecule ligands that interact and stabilize GPCRs are crucial for solving GPCR crystal structures. All of the current crystallized GPCRs were determined with the help of small molecule ligands. Of these structures, two class B GPCRs (GCGR and CRF$_1$R) possess the most abundant small molecule ligands in this family[12, 13]. Small molecules facilitate the folding of GPCRs, establishing a stable state for recognition and receptor binding. Additionally, the unique properties of small molecules, as opposed to peptidic ligands, allow them to play key roles in the stabilization of GPCRs, including in aqueous solubility and hydrogen bonding. Clearly, the structural information gained from GPCRs promotes small molecule drug discovery such that structure-based virtual screening and drug design have now become an indispensable component of this process. Indeed, in the past decade, a number of SAR studies on class A GPCRs resulted in breakthrough discoveries on how these receptors look and work. These studies greatly increased our understanding of the molecular mechanisms of receptor binding and activation and have enabled structure-

Table 2. Small molecule modulators for glucagon receptors.

No	Chemical structure	Bioactivity	Reference
9		Antagonist	[64]
10		Antagonist	[64]
11		Antagonist	[64]
12 (L-168049)		Selective antagonist IC_{50}= 3.7 nmol/L	[65]
13		Antagonist IC_{50}= 181 nmol/L	[66]
14		Antagonist IC_{50}= 89 nmol/L	[66]
15		Selective antagonist	[67]

(To be continued)

No	Chemical structure	Bioactivity	Reference
16		Selective antagonist	[67-68]
17		Selective antagonist	[69]
18 (SCH 900822)		Selective antagonist	[69]
19 (MK-0893)		Selective and competitive antagonist	[70]

based drug design[71].

Class B GPCRs have large soluble N-terminal domains along with 7-TMDs. Using the ECD structures of CRF$_1$R and corticotropin-releasing factor receptor 2α (CRF$_{2α}$R) with their four cognate ligands, Pal *et al* elucidated the structural basis of the differential ligand binding properties of these two receptors, suggesting that CRF$_1$R Glu-104 and CRF$_{2α}$R Pro-100 are responsible for the selective recognition of Ucn2 and Ucn3, respectively, by CRF$_{2α}$R[23]. In the glucagon subfamily, the ECD structures of GCGR and GLP-1R in complex with antibodies or natural ligands have been reported[72], showing that ligand specificity is primarily associated with the ECD while secondary recognition and signaling are mediated through the 7-TMDs[73-76]. The GLP-1R ECD structures in complex with

exendin$_{9-39}$ and GLP-1 clearly indicate the differences in molecular recognition between exendin-4 and GLP-1 that result in the higher binding affinity of exendin-4[26]. The GCGR ECD structure was solved using a blocking antibody that targeted the ECD as an inverse agonist, which suggests that the ECD negatively regulates receptor activity independent of ligand binding. For the TMD structure, a particularly challenging problem is that there is a lack of small molecule compounds to stabilize the receptors[13]. Clearly, the crystal structure of full-length GLP-1R will be a cornerstone for understanding the binding mechanism and for the design and development of small molecule ligands[40, 72, 77].

Using the GCGR antagonist NNC0640, a derivative of compound **11**, we were able to determine the 7-TMD crystal

structure of human GCGR, thereby providing essential information for designing safer and more efficacious medications to combat metabolic disorders[12, 68, 69]. This breakthrough was made in parallel with the determination by a British team of the structure of CRF$_1$R bound to the antagonist CP-376395[13]. Both receptors are stabilized by truncated TMDs and fused to helper proteins. It is known that class B GPCRs share 7-TMD arrangements and alignments that are similar to those of class A GPCRs. A common feature of these two class B receptors is the existence of a deep ligand-binding pocket. They also show the following crucial differences: 1) GCGR has a long helix 8 at the C-terminus, whereas crystallized CRF$_1$R was truncated after TM7; 2) at the GCGR N-terminus, TM1 extends a 'stalk' into the extracellular space; and 3) the CRF$_1$R antagonist binds deep in the cavity and is distant from the orthosteric site, whereas the GCGR structure did not co-crystallize with NNC0640[11].

Combining previous structural information on ECD-ligand complexes with the GCGR and CRF$_1$R TMD structures allowed us to construct a full receptor-ligand model, which accounted for the extensive interactions between peptide ligands and extracellular loops as well as the residues deep in the helical bundle, as reflected by mutagenesis and photo-cross-linking studies with different class B GPCRs[78]. The knowledge accumulated thus far offers a useful platform for the eventual determination of a full-length class B GPCR-ligand complex.

Conclusion
The development of small molecule ligands and the determination of crystal structures of glucagon subfamily members have increased our understanding of the molecular mechanisms governing receptor recognition and activation. Such insights are invaluable for the design and/or discovery of novel therapeutic agents targeting class B GPCRs.

Acknowledgements

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