A quest for connections : ligands for the HCA2, adenosine A3 and GPR88 receptors

Blad, C.C.

Chapter 1

General introduction

G protein-coupled receptors in health and disease

G protein-coupled receptors (GPCRs), alternatively called 7-TM receptors (for 7 transmembrane domains), comprise a large family of eukaryotic membrane proteins. When a GPCR is activated, for example by a hormone or a neurotransmitter, it transfers the message to intracellular signaling cascades. The most well-known of these cascades involve G protein activation, but recently it has become clear that other possible signaling mechanisms exist. In the end, these GPCR signals mediate vital functions of the human body, such as perception of the world around us, communication between the brain and other parts of the body, energy storage or mobilization, movement and fertility.

Table 1. Examples of drugs acting on GPCRs. Adapted from [5].

GPCR	Drug	Indication
β_2 adrenergic	albuterol	asthma
angiotensin AT_1	losartan	hypertension
calcitonin	calcitonin	osteoporosis
dopamine D_2	haloperidol	schizophrenia
gonadotropin-releasing factor	goserelin	cancer
histamine H2	ranitidine	ulcer
serotonin 1D	sumatriptan	migraine
leukotriene	pranlukast	allergy, asthma
μ opioid	morphine	pain

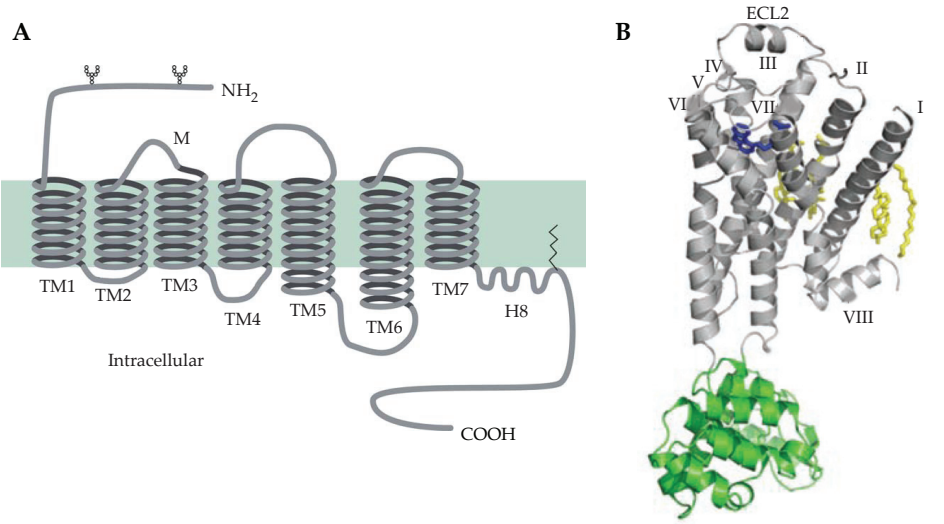

Figure 1. Common secondary structure of GPCRs. **A**: Schematic representation of a GPCR, indicating transmembrane domains 1-7 (TM1-7), the α-helical domain termed helix 8 (H8), a palmitoylation site next to it (zigzag line) and two potential glycosylation sites in the N-terminal tail (Y shapes). From [6]. **B**. High-resolution crystal structure of the β_2 adrenoceptor bound to carazolol (blue) together with the stabilizing T4 lysozyme protein (green) in place of the third intracellular loop. The helices that are shown next to each other in A form a barrel-like arrangement here, which is probably the form of the protein in vivo. The helices are indicated in roman numerals, including helix VIII in the C-terminal region. A small helix in extracellular loop 2 (ECL2), which may be typical for this receptor subtype, is also clearly visible. From [7].

A total of 799 verified human GPCRs are known, thus representing about 3% of all genes in our genome [1]. These receptors bind to a large variety of ligands including small organic compounds, lipids, peptides, proteins and even photons. Approximately 50% of the GPCR repertoire is dedicated to olfaction.

From a pharmacological point of view, GPCRs are interesting drug targets because they are implicated in many different pathophysiological processes. Furthermore, it is relatively straightforward to design molecules that interact with GPCRs and this type of drugs are successfully used in the clinic [2]. An estimated 30 to 40% of all available drugs target GPCRs, with annual sales of over 65 billion dollars [3]. Some examples are given in table 1. The variety of indications, for which these drugs are used, illustrates the omnipresence of GPCRs in the human body.

On a molecular level, all GPCRs have a common secondary structure. Their most striking features are the seven hydrophobic membrane-spanning alpha-helices (TM1-7) [4]. Extracellular and intracellular loops (EL and IL) connect the helices, and an extracellular N-terminal domain and intracellular C-terminus complete the protein (see figure 1A). The TM helices have been shown to form a barrel-like tertiary structure in the membrane, with TM1 and TM7 in close proximity (figure 1B).

GPCR classification

Several classifications have been proposed for the GPCR superfamily on the basis of primary structure, endogenous ligand specificity and species source. In a classical system by Kolakowski, the GPCRs are segregated into seven families or classes (A-F and O) [8]. A modern version of this system is used in the GPCRDB database www.gpcr.org [9], distinguishing the following classes: A: rhodopsin-like, B: secretin-like, C: metabotropic glutamate-like, D: pheromone receptors, E: cAMP receptors and F: frizzled/smoothened. After the human genome sequence became available, a slightly different system called GRAFS was proposed [10-12]. In this system, the classes are named Glutamate, Rhodopsin, Adhesion, Frizzled and Secretin, plus a recent addition, Taste2. Thus, class B from the A-F system is divided in the Adhesion and Secretin classes, which reflects the inherent differences between these receptor clusters. Classes A (Rhodopsin), B (Secretin&Adhesion) and C (Glutamate) are most important in mammalian physiology. Of these, class A is by far the largest and most diverse. It contains 672 receptors, which includes 388 olfactory receptors [13]. The GPCRs that will be discussed in this thesis all belong to the class A, rhodopsin-like, GPCR family.

Properties of Rhodopsin-like GPCRs

Although the class A receptors are highly diverse in sequence and ligand binding properties, certain residues in the TM regions are conserved (see figure 2). Most striking are the microdomains D/ERY (TM3), CWxP (TM6) and NPxxY (TM7). Furthermore, two conserved cysteine residues are present in the extracellular domain, at the end of TM3 and in extracellular loop 2. They are thought to form a disulfide bridge in most class A GPCRs, which may be crucial to structural integrity and receptor function (see for example [14]).

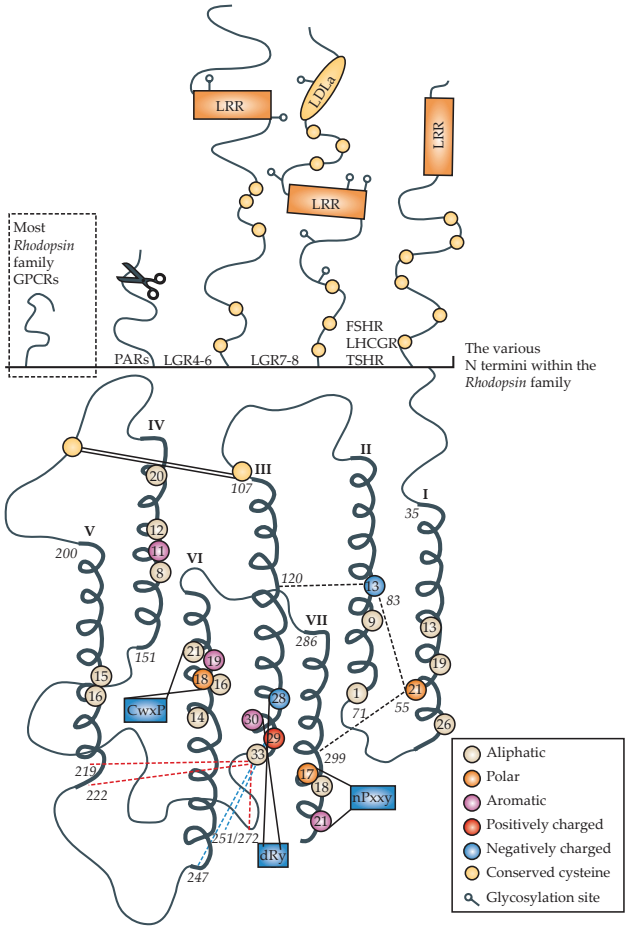

Figure 2. Conserved features and structural motifs within the Class A, rhodopsin-like, GPCRs. The lower part of the figure shows the conserved residues from eight diverse class A GPCRs. Conserved residues are shown as circles colour-coded for amino acid properties. In the blue boxes conserved class A motifs are indicated with uppercase letters for completely conserved positions, lowercase letters for well-conserved positions (>50%) and x for variable positions. Conserved cysteine residues are shown in yellow and the disulphide bridge between EL1 and EL2, which is conserved in most GPCRs, is indicated as two lines. Dashed black lines show hydrogen bonds within bovine rhodopsin whereas dashed blue lines (from DRY to 247 and 251) show the postulated ionic lock, which is thought to keep the receptor in the inactive state. Dashed red lines display Van der Waals interactions within the β_2-adrenoceptor model. In the upper part of the figure different N-termini of class A GPCRs are shown. The scissors indicate the cleavage site for the protease-activated receptors (PARs). From [13].

The position of the ligand binding site varies among class A receptors. Small ligands (such as biogenic amines, nucleosides, eicosanoids, lysophosphatidic acid and sphingosine-1-phosphate) bind in a so-called TM cavity, formed by the seven TM alpha helices ([15] and references therein). The ligands for the hydroxy-carboxylic acid receptors and the adenosine receptors, which are described in the present work, fall in this category. Larger ligands, such as peptides and glycoprotein hormones, interact with the N-terminus and/or the extracellular loops, and in some cases also with the outer portions of the transmembrane helices. Examples are oxytocin, vasopressin, opioids, thyroid-stimulating hormone (TSH) and follicle-stimulating hormone (FSH) [16].

High-resolution GPCR structures

Recently, some of the most important advances in the field have been made using X-ray crystallography. Structural analysis of GPCR molecules is very challenging and until recently, only the structure of rhodopsin was known [17-19]. In the last four years crystallization efforts finally paid off with the elucidation of high resolution crystal structures of squid rhodopsin, the β_2 adrenergic receptor (β_2AR), the β_1AR, the A_{2A} adenosine receptor (for a review see [20], see also figure 1), and most recently, the CXCR4 receptor [21], the dopamine D_3 receptor [22] and the histamine H_1 receptor [23]. These receptors were all stabilized in the inactive state, since this facilitates crystallization. For (rhod)opsin, the active apoprotein was also crystallized [24-25]. In 2011, active, transmitter-bound GPCRs have finally been crystallized, namely rhodopsin [26-27], the human β_2AR [28] and the adenosine A_{2A} receptor [29-30]. The newest structure of activated β_2AR is even complexed with a G_s protein [31]. This new (relative) wealth of structural information has increased our understanding of the structure of the specific GPCRs, the position of the ligand binding pockets and the orientation of the ligands in those pockets. The structures of the different receptors are remarkably similar, confirming that the secondary and tertiary structure of GPCRs is highly conserved, although there are, of course, subtle differences. For example, the binding pocket of the antagonist ZM241385 in the A_{2A} receptor was not as was anticipated on the basis of the β_2 adrenergic crystal structure with its antagonist carazolol. Therefore, care should be taken when homology models are used to predict the structure of a receptor that has not been crystallized yet. Comparison of active and inactive structures suggests that binding of an agonist results in conserved rearrangements near the binding site, which then propagate through the transmembrane domains to yield an active state. Binding of a G protein or substitute seems necessary to stabilize a fully active state [32].

Receptor activation leads to G protein signaling

GPCRs form an important connection between the outside and the inside of the mammalian cell. When an endogenous agonist like a hormone or a neurotransmitter binds to a GPCR, a conformational change occurs in the receptor protein, which commonly leads to the activation of a G protein (guanine nucleotide-binding protein) [33]. The G protein α subunit will release GDP from its GTPase catalytic site and bind GTP. This causes the G protein to dissociate from the GPCR, and divide into the α subunit and the $\beta\gamma$ complex. Both parts of the G protein can activate downstream effectors. The main Gα families are Gα_s, G$\alpha_{i/0}$ and Gα_q. Gα_s stimulates adenylate cyclase, thus stimulating cAMP production, whereas G$\alpha_{i/0}$ has the opposite effect, inhibiting adenylyl cyclase. Gα_q can stimulate phospholipase C, which causes an increase in cytoplasmic calcium concentrations. G$\beta\gamma$ dimers can activate phosphoinositide 3-kinases, which in turn activate other proteins, including MAP-kinases. Interestingly, these mitogen-activated protein kinases can also be activated by GPCRs via other, G protein-independent pathways [34]. When activated, they can influence gene expression. Other effects can also occur; the skin flushing side effect of drugs acting on the hydroxy-carboxylic acid receptor 2 (HCA_2) is possibly mediated by this pathway (see

chapter 2 and 3). The Gα subunit is deactivated when it converts GTP into GDP in its intrinsic GTPase domain. Gβγ dimers are deactivated when they bind a Gα subunit again.

The next step: internalization and arrestins

After a receptor has been activated, it is in many cases removed from the cell membrane. This internalization process is often (but not always) mediated by arrestins, which act as scaffold proteins that assemble a protein complex at the intracellular face of the receptor. Once inside the cell, the receptor protein can be broken down or recycled to the membrane after removal of the bound agonist [35-36]. Recruitment of arrestins to a receptor protein can also result in arrestin-mediated signaling, leading for example to the MAP-kinase activation mentioned above [37-38].

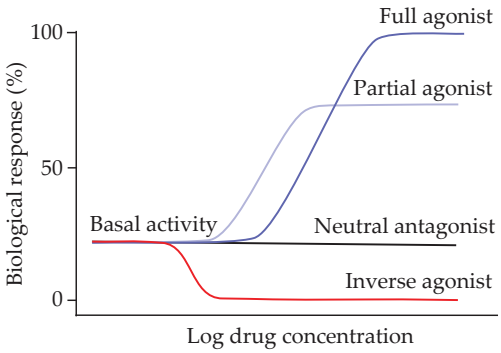

Figure 3. Dose-response curves of ligands with different efficacy. Many GPCRs also display some basal activity in absence of a ligand. From: [20].

Different ligands have different effects

Next to agonists, GPCR ligands exist that de-activate the receptor (inverse agonists), as well as ligands that block the binding site but do not change the receptor activation state (neutral antagonists) (figure 3). We can also distinguish full agonists, which fully activate the receptor, and partial agonists, which cannot cause full activation even at concentrations that fully occupy the receptor. This last type of ligands for the HCA_2 receptor is investigated in chapter 3. The existence of partial agonists suggests that GPCRs are not simply on-off switches. Rather, the receptor protein is now thought to exist in a range of conformational states. A bound ligand stabilizes a particular subset of states, and this determines which of all possible signaling pathways will be influenced, and also with which efficacy. In this more complex view of the receptor protein, a ligand is not simply an agonist, antagonist or inverse agonist, but can be, for example, an inverse agonist for the G_s pathway, and at the same time an agonist for the arrestin pathway [38-39]. In fact, this extreme example of 'biased signaling' has been demonstrated for the β_2-adrenoceptor, where propranolol functioned as an inverse agonist on the G_s protein and its downstream pathways, but as a partial agonist on (most likely arrestin-mediated) activation of extracellular signal-regulated kinase (ERK)1/2 [40]. Thus, one ligand binding to one receptor can have a whole spectrum of effects. A slightly different ligand may prompt the downstream cascades of

the same receptor in a different manner. These phenomena, termed ligand texture and ligand-directed signaling, have only recently been recognized [39]. Ligand bias on the HCA_2 receptor is discussed in chapters 2 and 3 of this thesis.

Manipulating downstream signaling

Understanding of the intracellular signaling cascades and how GPCRs stimulate them made it possible to bend these pathways to suit research purposes. For example, in immortalized cell lines expressing high levels of the chimeric G protein G_{qi5}, all GPCRs that normally interact with G_i proteins are forced to signal through the G_q pathway, leading to an easily detectable increase in intracellular calcium concentration [41-42]. G_{qi5} is identical to the G_q protein, except for five C-terminal residues which are exchanged for their G_i counterparts. These five amino acids determine the interaction with the receptor. Many variations of this strategy exist and are employed in the search for new GPCR ligands, often in a high-throughput screening setup where thousands of compounds can be tested per day. One example of such a screen is reported in chapter 7 of this work.

Allosteric modulators

A special class of GPCR ligands is formed by allosteric modulators. These compounds bind to the receptor at a site distinct from the so-called orthosteric binding site, where the endogenous ligand binds. Binding of the modulator can influence the conformation of the receptor and modify the affinity and/or efficacy of orthosteric ligands. Some allosteric modulators also have intrinsic efficacy themselves and can activate the receptor without binding of an orthosteric agonist. Allosteric modulators can be promising drug candidates since they may be more specific and have less side effects. Allosteric binding sites of synthetic ligands are, in principle, not evolutionarily conserved, which makes it less likely for an allosteric modulator to have affinity for related GPCRs [43]. Furthermore, an allosteric enhancer that makes the endogenous agonist more potent could boost the natural signal without constantly activating the receptor. This can be an important therapeutic advantage because it improves the timing and localization of receptor activation. Two allosteric GPCR modulators are currently on the market: the calcimimetic cinacalcet, which is a positive allosteric enhancer of Ca^{2+}-sensing receptors, and the anti-HIV drug maraviroc, an allosteric inhibitor of chemokine receptor CCR_5 [44]. In chapter 5 of this thesis a number of new allosteric modulators for the HCA_2 receptor are presented.

Orphan receptors and the quest for ligands

As mentioned above, a total of 799 human GPCRs have been identified, of which 369 are presumably non-sensory GPCRs (not involved in taste, vision or smell). Approximately 100 to 140 of these potential drug targets are still 'orphans', with no known endogenous ligand and in most cases no known function [1, 45].

Classic and reverse pharmacology

The first GPCRs that were purified and cloned in the 1980s, were receptors for known signaling molecules (adrenalin and noradrenalin) that had been studied for decades (for

a review see [46]). This approach, where receptors are identified to match the known signaling molecules, has later been termed the classic pharmacology approach (see figure 4, left scheme). After the first expression cloning of the β_2-adrenoceptor in 1986 [47], and the finding that this receptor probably shared the 7 transmembrane domain topology of rhodopsin, pioneers of GPCR research soon suspected that this feature was common to many receptors signaling through G proteins. The rapid homology cloning of many other GPCRs, including those for acetylcholine, serotonin and the neuropeptide substance K, confirmed their hypothesis [48-49]. Some of the receptors that were cloned did not have a known ligand, but in many cases they were readily paired to one of the many 'orphan' signaling molecules. However, in the 1990s the number of cloned receptors had vastly increased due to the advent of PCR techniques, and the number of known signaling molecules that were not yet coupled to a receptor was dwindling. This imbalance reversed the roles of transmitter and receptor in research: known orphan receptors were used to fish for novel signaling molecules, instead of the other way around. The term reverse pharmacology is used for this approach. In this thesis, it was applied on the orphan receptor GPR88 (chapter 7).

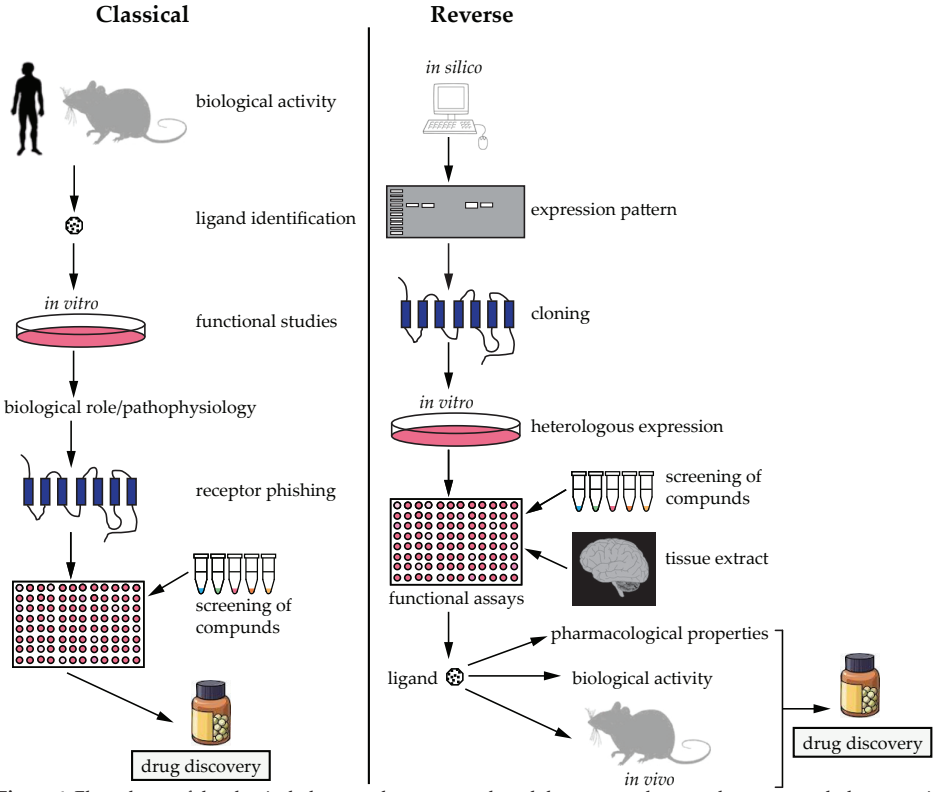

Figure 4. Flow charts of the classical pharmacology approach and the reverse pharmacology approach that arose in the 1990s, mostly due to advances in homology cloning. Adapted from [5, 50].

The differences between the classical approach and the reverse pharmacology approach are illustrated in figure 4. In reverse pharmacology, an orphan GPCR is first identified

by homology cloning using molecular biology techniques, or, since the sequence of the human genome is known, in silico. The GPCR of interest is then expressed in a heterologous cell system, followed by screening against a compound collection or purified tissue extracts for receptor activation. The use of tissue extracts increases the chances of finding the endogenous ligand. However, important technical difficulties are associated with the latter strategy, including low signal-to-noise ratio and failure to isolate the ligand from an active extract. Therefore, synthetic compound collections are often used instead. The first receptors that were deorphanized by reverse pharmacology were the 5-HT_{1A} serotonin receptor and the dopamine D_2 receptor [51-52]. In total, the application of the reverse pharmacology approach led to the 'deorphanization' of more than 150 GPCRs, coupling them to approximately 75 endogenous ligands. Many of these ligands were novel

Table 2. GPCRs listed as orphan receptors by IUPHAR with (putative) ligands identified from the literature.

Name	Pseudonyms	Ligand(s)	References
CCRL2	CRAM	CCL5 (RANTES), CCL19, chemerin	[53-56]
CMKRL1	ChemR23	chemerin, resolvin E1 (RvE1), SIV/HIV-1 coreceptor	[57-60]
CMKOR1	CXCR7, RDC1	CXCL12/SDF-1α	[61-62]
GPR1		chemerin; glucose/sucrose (S. cerevisiae), HIV/SIV coreceptor	[63-65]
GPR3		sphingosine 1-phosphate (S1P)	[66]
GPR4		protons, lysolipids (?)	[67-69]
GPR6		S1P (?)	[66, 70]
GPR12		tyrosol, S1P, sphingosylphosphorylcholine (SPC)	[66, 71-72]
GPR17		nucleotides, cysteinyl leukotriene (CysLT)	[73]
GPR18		N-arachidonoylglycine, Δ(9)-tetrahydrocannabinol	[70, 74-75]
GPR23	P2Y9, LPA4	lysophosphatidic acid (LPA)	[76]
GPR32		resolvin D1 (RvD1)	[77]
GPR34		lysophosphatidyl-L-serine	[78]
GPR35		kynurenic acid, 2-acyl lysophosphatidic acids	[79-80]
GPR37	PAEL	neuropeptide head activator	[81]
GPR39		Zn^{2+}	[82-83]
GPR55		lysophosphatidylinositol, cannabinoids	[84-85]
GPR63		S1P (?), dioleoylphosphatidic acid	[70, 86]
GPR65	TDAG8	protons, psychosine (?)	[87-88]
GPR68	OGR1	protons, sphingosylphosphorylcholine (?)	[68, 89-90]
GPR75		CCL5 (RANTES)	[91]
GPR84		medium-chain free fatty acids (FFA)	[92]
GPR92	GPR93, LPA5	LPA, farnesyl pyrophosphate, geranyl geranyl diphosphate	[93-96]
GPR119		oleoyl-lysophosphatidylcholine (OLPC), oleoylethanolamide (OEA), N-oleoyldopamine (OLDA)	[97-99]
GPR120		FFA	[100]
GPR132	G2A	protons, lysolipids (?), oxydized FFA (9-HODE, 11-HETE)	[101-103]
MAS1	Mas	Angiotensin 1-7, neuropeptide FF	[104-105]
MRGPRD		β-alanine	[106]
MrgprX1	MrgX1, SNSR4	BAM8-22, BAM22 (1-22) and related peptides	[107]
MrgprX2	MrgX2	corticostatin-14	[108]
OPN5	GPR99	photoreceptor (birds)	[109]
OXGR1	GPR99	α-ketoglutarate (2-oxoglutarate)	[110]
SUCNR1	GPR91	succinate	[110]

and unexpected signaling molecules, including calcium ions, trace amines, bile acids, kynurenic acid, protons, oleoylethanolamide, lysophosphatidylinositol, lysophosphatidic acid and a plethora of new neuropeptides.

Ligands recently proposed for class A orphans

In the receptor database of the International Union of Basic and Clinical Pharmacology (IUPHAR-db), 97 rhodopsin-like (class A) orphans are listed. However, a search of the recent literature yielded putative endogenous ligands for 33 of these receptors (see table 2). In some cases further studies may be needed, but we can assume that many of these receptors are now truly deorphanized. This shows that the deorphanization efforts still yield results. All but four of the new receptor-ligand pairs belong in 6 subgroups: proton/lysolipid (GPR4, GPR65, GPR68, G2A), chemokine (CCRL2, CMKRL1, CMKOR1, GPR1, GPR75), lipid mediators (GPR3, GPR6, GPR12, GPR23, GPR32, GPR34, GPR63, GPR92, GPR119), cannabinoid (GPR18, GPR55), Mas related (Mas, MRGPRD, MrgprX1, MrgprX2), and metabolic intermediates (GPR84, OXGR1, SUCNR1, GPR120). A number of these receptors seem to have more than one endogenous ligand. One could state that these receptors were not truly deorphanized when only one of the ligands was identified. Of course, it is impossible to prove that no additional ligands remain to be discovered for any given receptor. In chapter 6 of the current work, a new, possibly endogenous, ligand for the adenosine A_3 receptor is reported.

Deorphanized receptors and the regulation of food intake

Clearly, deorphanization of GPCRs has had a profound influence on our understanding of mammalian physiology. For example, particularly large advances have been made in our understanding of the regulation of food intake. Several novel neuropeptides have been discovered as ligands for orphan GPCRs, including leptin, ghrelin and orexin [for a review see 45]. Furthermore, several GPCRs have been shown to react to nutrients and metabolic intermediates. These receptors seem to function as sensors for food and metabolic status, which is an unexpected new role for GPCRs. Nutrient sensing receptors include the calcium sensing receptor, GPRC6A and the dimeric taste receptor complex T1R1/T1R3, which are promiscuous receptors for several L-α-amino acids and divalent cations, as well as the T1R2/T1R3 dimer, which responds to sugars and D-amino acids [111]. These receptors are expressed in taste tissue, the gastrointestinal tract, endocrine glands, adipose tissue, and/or kidney, where they regulate the release of hormones important for metabolism and the regulation of food intake. They can also influence gene expression, for example yielding an increase in the expression of nutrient transporters. Next to these class C receptors, several class A receptors are also involved in nutrient sensing. GPR92/93 expressed in the small intestine is activated by proteolytic degradation products and by lysophosphatidic acid [93-96]. Medium- and long-chain free fatty acids, from the hydrolysis of ingested fat and oil, activate the free fatty acid receptor 1 (FFA1) as well as GPR84 and GPR120 [92, 100, 112-114]. FFA1 is expressed in the islets of Langerhans in the pancreas and the gut, whereas GPR120 is expressed in the gut, in adipose tissue and in the lung. FFA1 activation stimulates the release of the hormone GLP-1 from the gut, a role which may be shared

by GPR120. Activated FFA1 also potentiates glucose-stimulated insulin release from the pancreas, and it might be involved in the toxic effect of chronic high circulating free fatty acid levels on pancreatic β cells [115]. GPR84 is expressed in immune cells and its role in physiology has not yet been elucidated. Short-chain free fatty acids, which are mainly fermentation products of carbohydrate fibers, are ligands of the FFA2 and FFA3 receptors [116-117]. FFA2 is primarily expressed in immune cells, and has been shown to play an important role in neutrophil recruitment during intestinal inflammation [118], and it is thought to be involved in leukocyte chemotaxis by sensing the presence of bacterial fermentation products. Additionally, it appears to be involved in energy homeostasis and appetite regulation [119], including the mediation of the anti-lipolytic effect of acetate and proprionate [120]. FFA3 is more widely expressed than FFA2, with the highest expression in adipose tissue, and more moderate levels in immune cells and tissues. It has been suggested that FFA3 mediates the effect of proprionate on leptin release [121]. Next to nutrients, intermediates in (energy) metabolism have also been identified as GPCR ligands, some of which were mentioned above (see table 2). More specifically, succinate and α-ketoglutarate (2-oxoglutarate), two intermediates from the citric acid cycle, are the endogenous ligands of GPR91 and GPR99, respectively, and the receptors are now named SUCNR1 and OXGR1 [110]. These receptors are predominantly expressed in the kidney. Succinate stimulates renin release via GPR91, which causes an increase in blood pressure. Intermediates of the β-oxidation process, which is upstream of the citric acid cycle, activate HCA_3 (GPR109B) [122]. The main ligand seems to be 3-hydroxy-octanoic acid. The same receptor has also been reported as a receptor for aromatic D-amino acids [123]. Two related receptors, HCA_2 (GPR109A) and HCA_1 (GPR81), are activated by the ketone body 3-hydroxybutyrate and by lactate, respectively [124-126]. Butyrate, a short-chain free fatty acid, may be an additional ligand for HCA_2 in the gut [127]. All three hydroxy-carboxylic acid receptors have an anti-lipolytic effect when activated. HCA_2 and HCA_3 are part of a negative feedback loop which keeps the release of fat stores in check under starvation conditions, whereas HCA_1 plays a role in the antilipolytic effect of insulin [review 128]. This family of hydroxy-carboxylic acid receptors is discussed in detail in chapter 2, and HCA_2 is the focus of chapters 3, 4 and 5.

Current challenges in deorphanization

After a peak in 2003 the deorphanization rate seems to be declining (see figure 5) [129]. This may be partly due to a shift in focus in the pharmaceutical industry. Many companies are currently faced with expiring patents of major blockbusters and increasing rules and regulations regarding safety, resulting in increased numbers of leads that fail to reach the market. The cost of compound screens for deorphanization is high and success not assured. Even if a ligand can be linked to an orphan receptor many years of additional R&D are needed to put a drug on the market. Less costly R&D programs could target the approximately 175 non-orphan GPCRs that are not currently targeted by drugs. Novel ligands, including allosteric modulators, or optimized versions of known ligands, can also be developed for the ~50 current GPCR drug targets. In the present climate pharmaceutical companies may more likely choose these R&D strategies.

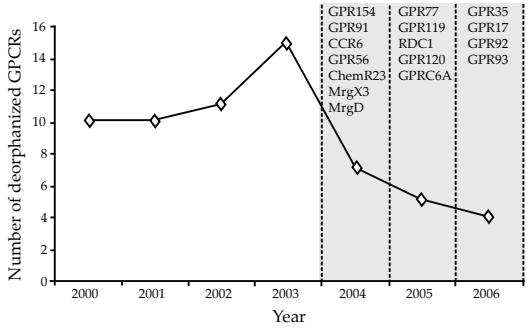

Figure 5. Declining rate of deorphanization since 2004. Adapted from [129].

Furthermore, a number of challenges complicate the identification of ligands for the remaining orphan GPCRs. Many of the remaining orphan GPCRs do not show high sequence homology to a cluster of GPCRs with similar ligands. When orphans are phylogenetically localized between two receptor subfamilies it may mean they bind ligands that share properties of both ligand families. Some other orphans, such as GPR88, do not show any significant homology to a known receptor subfamily. Purification of a known ligand type from tissue extracts is already a highly challenging task, and if the ligand properties are totally unknown another degree of complexity is added.

Why did traditional screening methods fail to bring to light the ligands for the remaining orphan GPCRs? It is possible that some of these orphans display non-traditional signaling, for example through G protein-independent signaling cascades. Traditional assays are

Table 3. Ligand-independent functions of orphan GPCRs. Adapted from [130].

GPCR	Orphan 7TM protein	Effect on function
$GABA_{B1}$	$GABA_{B2}$	Export to the cell surface and G protein coupling
DOR22a	DOR83b	Export and improvement of functionality
DOR43a	DOR83b	Export and improvement of functionality
T1R1	T1R3	Effect on receptor functionality and pharmacology
T1R2	T1R3	Effect on receptor functionality and pharmacology
MrgD	MrgE	Decrease of internalization; increase of ERK phosphorylation and intracellular $[Ca^{2+}]$
MT_1	GPR50	Loss of ligand binding and function
	ORF74	Constitutive activity responsible for oncogenic action of Herpesvirus 8
	UL33	Constitutive activity responsible for HCMV-related pathologies
	EBI2	Constitutive activity in Epstein-Barr virus-infected cells

DOR: *Drosophila* odorant receptors; EBI2: Epstein-Barr virus-induced receptor 2; ERK: extracellular signal-regulated kinase; HCMV: human cytomegalovirus; Mrg: Mas related gene; MT_1: melatonin receptor 1; Smo: Smoothened; T1R: taste receptor.

not equipped to identify this activation. The orphans may also need unknown interacting partners in order to function, for example another GPCR to form a functional heterodimer. In heterologous cell systems correct expression of functional GPCRs cannot always be obtained due to the absence of necessary protein partners.

Finally, some of the orphans may be 'real' orphans with no physiological ligand at all,

Table 4. Remaining class A orphan GPCRs from IUPHAR-db excluding the putatively liganded GPCRs shown in table 2. The receptors are classified according to a phylogenetic analysis by Joost and Methner [143]. The receptors at the bottom of the table were not included in this analysis.

Group	Orphan	Pseudonyms	Remarks
A03	GPR15	GPRF	
	GPR25		
A06	GPR22	GPRM	constitutive activity [144]
A08	MAS1L		
	MRGPRE		
	MRGPRF		
	MRGPRG	GPR169	
	MRGPRX3	SNSR1/2	
	MRGPRX4		
A09	GPR19	GPRJ	
	GPR50		
A10	LGR4	GPR48	
	LGR5	GPR49, GPR67	
	LGR6		
A11	GPR31	GPRV	
	GPR42		
	GPR82		
A12	GPR34	GPRy	
	GPR87		
A15	EBI2	GPR183	constitutive activity [140]
	P2RY5		
	P2RY8		
	P2RY10		
	GPR21	GPRL	
A18	GPR27	SREB1	
	GPR45		
	GPR52		
	GPR61		constitutive activity [145]
	GPR62		
	GPR78		constitutive activity [146]
	GPR85	SREB2	
	GPR88		
	GPR101		
	GPR173	SREB3	
	GPR20		constitutive activity [147]
	GPR26		constitutive activity [146]
	GPR33		pseudogene in most individuals [148]
	GPR37L1		
	GPR79		probable pseudogene [149]
	GPR83		
	GPR135		
	GPR139		
	GPR1		
	GPR142		
	GPR146		
	GPR148		
	GPR149		
	GPR150		
	GPR151	GalR4, GalRL	
	GPR152		
	GPR153		
	GPR160		
	GPR161		
	GPR162		
	GPR171		
	GPR174		
	GPR182		
	OPN3		
	TAAR2	GPR58	
	TAAR3		probable pseudogene in primates [150]
	TAAR4		probable pseudogene in primates [150]
	TAAR5		
	TAAR6		
	TAAR8		
	TAAR9		

that exert their function(s) through ligand-independent mechanisms. Some examples of orphan GPCRs with known ligand-independent activity are summarized in table 3 [130]. The top four 'orphan 7TM proteins' (indeed, they may not be receptors, nor G protein-coupled) in the table interact with the listed non-orphan GPCR to help it reach the cell surface (GABA, DOR) [131-133], change its functionality and/or signalling (DOR, T1R, Mrg) [131-132, 134-137], or inhibit its function (MT_1/GPR50) [138]. The bottom three orphans, all virus-encoded, do not need a ligand to function because they are active in the absence of a ligand [139-142]. Evolutionary analysis showing the presence or absence of evolutionary pressure on the protein as a whole, or on the predicted binding site, can be used to determine whether the protein is likely to be functional and whether it is likely to interact with a ligand in order to function.

Inventarisation of Class A orphan GPCRs

In table 4, all remaining class A (Rhodopsin-like) orphan receptors are shown, taken from the IUPHAR-db but not including the receptors listed in table 2. Some of the receptors display constitutive activity, which may indicate that these proteins do not need ligands to function. Others are likely to be pseudogenes in some or all individuals. Phylogenetic analysis showing sequence similarities between orphan and liganded receptors can give valuable clues where to start the quest for a ligand. In table 4 the orphan GPCRs are classified according to an analysis by Joost and Methner [143]. Another useful method to classify orphan receptors is using phylogenetic analysis of the residues predicted to line the ligand binding cavity, as was done by Surgand and colleagues [15]. This method yielded surprising results in the case of GPR88, which belongs to the Rhodopsin-like receptors on the basis of its full sequence, but was classified with the Glutamate-like receptors (class C) in this analysis.

Deorphanization of the 65 orphan GPCRs in table 4, and/or elucidation of their biological functions, could have a great impact on our understanding of mammalian physiology. Furthermore, keeping in mind that drugs on the market today target only ~50 GPCRs, understanding of these orphans may give rise to a whole range of new medicines.

Objectives and overview of this thesis

In the current work deorphanization and receptor-ligand pairing are a leading theme. I will describe studies on three GPCRs: the hydroxy-carboxylic acid receptor 2 (HCA_2), the orphan receptor GPR88 and the adenosine A_3 receptor (A_3R).

HCA_2 is a recently deorphanized GPCR that is of great interest as a drug target. In fact, one of its ligands, nicotinic acid, has been used as an anti-dyslipidemia drug for over 50 years. As described above, the endogenous ligand of HCA_2 is 3-hydroxybutyrate, which acts as a negative feedback signal to preserve fat tissue during times of starvation. The biological and pharmacological roles of the HCA_2 receptor and its two close family members HCA_1 and HCA_3 are reviewed in chapter 2. In my research, I explored the signaling cascades that are influenced upon HCA_2 activation by synthetic and endogenous agonists (chapter 3). In the same chapter I describe the in vivo effects of two partial agonists for HCA_2.

Furthermore, I investigated the binding of synthetic compounds to HCA_2 both in an orthosteric and in an allosteric manner (chapter 4 and 5).

As I have mentioned above, a receptor does not necessarily have only one unique ligand. For the adenosine A_3 receptor, there are indications that it binds an additional ligand next to its canonical agonist adenosine. This ligand could be involved in the resistance of muscle tissue to tumor metastases. I attempted to identify this elusive ligand, and identified the antiproliferative compound N^6-isopentenyl adenosine as an A_3R ligand that may or may not be endogenous (chapter 6).

GPR88 is an orphan GPCR expressed predominantly in two brain regions: the striatum and the central extended amygdala. GPR88 could be of major interest therapeutically, and I set out to identify a synthetic, if not endogenous, agonist for this receptor, screening a large number of compounds in a functional assay (chapter 7).

In this thesis, a recently discovered and deorphanized receptor is further investigated, expanding the ligand repertoire for two binding sites on the receptor; a new ligand is proposed for a GPCR long since paired to its ligand; and a ligand screen on an orphan GPCR is described, clearly identifying the challenges of such an operation. This research into the 'simple' interaction between small molecules and membrane proteins gives insights into human physiology and the mechanism of action of (future) drugs, and opens new horizons for pharmacotherapy.

References

1. Gloriam, D.E., R. Fredriksson, and H.B. Schioth, *The G protein-coupled receptor subset of the rat genome.* BMC Genomics, 2007. **8**: p. 338.
2. Russ, A.P. and S. Lampel, *The druggable genome: an update.* Drug Discov Today, 2005. **10**(23-24): p. 1607-10.
3. Overington, J.P., B. Al-Lazikani, and A.L. Hopkins, *How many drug targets are there?* Nat Rev Drug Discov, 2006. **5**(12): p. 993-6.
4. Baldwin, J.M., *The probable arrangement of the helices in G protein-coupled receptors.* EMBO J, 1993. **12**(4): p. 1693-703.
5. Stadel, J.M., S. Wilson, and D.J. Bergsma, *Orphan G protein-coupled receptors: a neglected opportunity for pioneer drug discovery.* Trends Pharmacol Sci, 1997. **18**(11): p. 430-7.
6. Deupi, X. and B. Kobilka, *Activation of G protein-coupled receptors.* Adv Protein Chem, 2007. **74**: p. 137-66.
7. Cherezov, V., et al., *High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor.* Science, 2007. **318**(5854): p. 1258-65.
8. Kolakowski, L.F., Jr., *GCRDb: a G-protein-coupled receptor database.* Receptors Channels, 1994. **2**(1): p. 1-7.
9. Horn, F., et al., *GPCRDB information system for G protein-coupled receptors.* Nucleic Acids Res, 2003. **31**(1): p. 294-7.
10. Fredriksson, R., et al., *The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints.* Mol Pharmacol, 2003. **63**(6): p. 1256-72.

11. Schiöth, H.B. and R. Fredriksson, *The GRAFS classification system of G-protein coupled receptors in comparative perspective.* Gen Comp Endocrinol, 2005. **142**(1-2): p. 94-101.
12. Tyndall, J.D. and R. Sandilya, *GPCR agonists and antagonists in the clinic.* Med Chem, 2005. **1**(4): p. 405-21.
13. Lagerstrom, M.C. and H.B. Schioth, *Structural diversity of G protein-coupled receptors and significance for drug discovery.* Nat Rev Drug Discov, 2008. **7**(4): p. 339-57.
14. Cook, J.V. and K.A. Eidne, *An intramolecular disulfide bond between conserved extracellular cysteines in the gonadotropin-releasing hormone receptor is essential for binding and activation.* Endocrinology, 1997. **138**(7): p. 2800-6.
15. Surgand, J.S., et al., *A chemogenomic analysis of the transmembrane binding cavity of human G-protein-coupled receptors.* Proteins, 2006. **62**(2): p. 509-38.
16. Massotte, D. and B. Kieffer, *Structure-function relationships in G protein-coupled receptors: ligand binding and receptor activation*, in *G protein-coupled receptors Handbook*, L. Devi, Editor. 2005, Humana Press. p. 2-32.
17. Palczewski, K., et al., *Crystal structure of rhodopsin: A G protein-coupled receptor.* Science, 2000. **289**(5480): p. 739-45.
18. Li, J., et al., *Structure of bovine rhodopsin in a trigonal crystal form.* J Mol Biol, 2004. **343**(5): p. 1409-38.
19. Okada, T., et al., *The retinal conformation and its environment in rhodopsin in light of a new 2.2 A crystal structure.* J Mol Biol, 2004. **342**(2): p. 571-83.
20. Rosenbaum, D.M., S.G. Rasmussen, and B.K. Kobilka, *The structure and function of G-protein-coupled receptors.* Nature, 2009. **459**(7245): p. 356-63.
21. Wu, B., et al., *Structures of the CXCR4 chemokine GPCR with small-molecule and cyclic peptide antagonists.* Science. **330**(6007): p. 1066-71.
22. Chien, E.Y., et al., *Structure of the human dopamine D3 receptor in complex with a D2/D3 selective antagonist.* Science, 2010. **330**(6007): p. 1091-5.
23. Shimamura, T., et al., *Structure of the human histamine H1 receptor complex with doxepin.* Nature, 2011. **475**(7354): p. 65-70.
24. Park, J.H., et al., *Crystal structure of the ligand-free G-protein-coupled receptor opsin.* Nature, 2008. **454**(7201): p. 183-7.
25. Scheerer, P., et al., *Crystal structure of opsin in its G-protein-interacting conformation.* Nature, 2008. **455**(7212): p. 497-502.
26. Choe, H.W., et al., *Crystal structure of metarhodopsin II.* Nature, 2011. **471**(7340): p. 651-5.
27. Standfuss, J., et al., *The structural basis of agonist-induced activation in constitutively active rhodopsin.* Nature, 2011. **471**(7340): p. 656-60.
28. Rasmussen, S.G., et al., *Structure of a nanobody-stabilized active state of the beta(2) adrenoceptor.* Nature, 2011. **469**(7329): p. 175-80.
29. Lebon, G., et al., *Agonist-bound adenosine A2A receptor structures reveal common features of GPCR activation.* Nature, 2011. **474**(7352): p. 521-5.
30. Xu, F., et al., *Structure of an agonist-bound human A2A adenosine receptor.* Science,

2011. **332**(6027): p. 322-7.

31. Rasmussen, S.G., et al., *Crystal structure of the beta2 adrenergic receptor-Gs protein complex.* Nature, 2011. **477**(7366): p. 549-55.
32. Deupi, X. and J. Standfuss, *Structural insights into agonist-induced activation of G-protein-coupled receptors.* Curr Opin Struct Biol, 2011. **21**(4): p. 541-51.
33. Patrick, G.L., ed. *An introduction to medicinal chemistry*. 2nd ed. ed. 2001, Oxford.
34. Strange, P.G., *Signaling mechanisms of GPCR ligands.* Curr Opin Drug Discov Devel, 2008. **11**(2): p. 196-202.
35. Ferguson, S.S., *Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling.* Pharmacol Rev, 2001. **53**(1): p. 1-24.
36. Pierce, K.L., R.T. Premont, and R.J. Lefkowitz, *Seven-transmembrane receptors.* Nat Rev Mol Cell Biol, 2002. **3**(9): p. 639-50.
37. Sun, Y., D. McGarrigle, and X.Y. Huang, *When a G protein-coupled receptor does not couple to a G protein.* Mol Biosyst, 2007. **3**(12): p. 849-54.
38. Violin, J.D. and R.J. Lefkowitz, *Beta-arrestin-biased ligands at seven-transmembrane receptors.* Trends Pharmacol Sci, 2007. **28**(8): p. 416-22.
39. Urban, J.D., et al., *Functional selectivity and classical concepts of quantitative pharmacology.* J Pharmacol Exp Ther, 2007. **320**(1): p. 1-13.
40. Azzi, M., et al., *Beta-arrestin-mediated activation of MAPK by inverse agonists reveals distinct active conformations for G protein-coupled receptors.* Proc Natl Acad Sci U S A, 2003. **100**(20): p. 11406-11.
41. Conklin, B.R., et al., *Substitution of three amino acids switches receptor specificity of Gq alpha to that of Gi alpha.* Nature, 1993. **363**(6426): p. 274-6.
42. Coward, P., et al., *Chimeric G proteins allow a high-throughput signaling assay of Gi-coupled receptors.* Anal Biochem, 1999. **270**(2): p. 242-8.
43. May, L.T., et al., *Allosteric modulation of G protein-coupled receptors.* Annu Rev Pharmacol Toxicol, 2007. **47**: p. 1-51.
44. Burford, N.T., et al., *Strategies for the identification of allosteric modulators of G-protein-coupled receptors.* Biochem Pharmacol, 2011. **in press**.
45. Chung, S., T. Funakoshi, and O. Civelli, *Orphan GPCR research.* Br J Pharmacol, 2008. **153 Suppl 1**: p. S339-46.
46. Lefkowitz, R.J., *Historical review: a brief history and personal retrospective of seven-transmembrane receptors.* Trends Pharmacol Sci, 2004. **25**(8): p. 413-22.
47. Dixon, R.A., et al., *Cloning of the gene and cDNA for mammalian beta-adrenergic receptor and homology with rhodopsin.* Nature, 1986. **321**(6065): p. 75-9.
48. Hall, Z.W., *Three of a kind: the beta-adrenergic receptor, the muscarinic acetylcholine receptor, and rhodopsin.* Trends Neurosci, 1987. **10**: p. 99-101.
49. Masu, Y., et al., *cDNA cloning of bovine substance-K receptor through oocyte expression system.* Nature, 1987. **329**(6142): p. 836-8.
50. Lecca, D. and M.P. Abbracchio, *Deorphanisation of G protein-coupled receptors: A tool to provide new insights in nervous system pathophysiology and new targets for psycho-active drugs.* Neurochem Int, 2008. **52**(3): p. 339-51.
51. Bunzow, J.R., et al., *Cloning and expression of a rat D2 dopamine receptor cDNA.*

Nature, 1988. **336**(6201): p. 783-7.
52. Fargin, A., et al., *The genomic clone G-21 which resembles a beta-adrenergic receptor sequence encodes the 5-HT1A receptor.* Nature, 1988. **335**(6188): p. 358-60.
53. Hartmann, T.N., et al., *Human B cells express the orphan chemokine receptor CRAM-A/B in a maturation-stage-dependent and CCL5-modulated manner.* Immunology, 2008. **125**(2): p. 252-62.
54. Leick, M., et al., *CCL19 is a specific ligand of the constitutively recycling atypical human chemokine receptor CRAM-B.* Immunology, 2010. **129**(4): p. 536-46.
55. Yoshimura, T. and J.J. Oppenheim, *Chemerin reveals its chimeric nature.* J Exp Med, 2008. **205**(10): p. 2187-90.
56. Zabel, B.A., et al., *Mast cell-expressed orphan receptor CCRL2 binds chemerin and is required for optimal induction of IgE-mediated passive cutaneous anaphylaxis.* J Exp Med, 2008. **205**(10): p. 2207-20.
57. Arita, M., et al., *Stereochemical assignment, antiinflammatory properties, and receptor for the omega-3 lipid mediator resolvin E1.* J Exp Med, 2005. **201**(5): p. 713-22.
58. Meder, W., et al., *Characterization of human circulating TIG2 as a ligand for the orphan receptor ChemR23.* FEBS Lett, 2003. **555**(3): p. 495-9.
59. Samson, M., et al., *ChemR23, a putative chemoattractant receptor, is expressed in monocyte-derived dendritic cells and macrophages and is a coreceptor for SIV and some primary HIV-1 strains.* Eur J Immunol, 1998. **28**(5): p. 1689-700.
60. Wittamer, V., et al., *Specific recruitment of antigen-presenting cells by chemerin, a novel processed ligand from human inflammatory fluids.* J Exp Med, 2003. **198**(7): p. 977-85.
61. Balabanian, K., et al., *The chemokine SDF-1/CXCL12 binds to and signals through the orphan receptor RDC1 in T lymphocytes.* J Biol Chem, 2005. **280**(42): p. 35760-6.
62. Burns, J.M., et al., *A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell adhesion, and tumor development.* J Exp Med, 2006. **203**(9): p. 2201-13.
63. Barnea, G., et al., *The genetic design of signaling cascades to record receptor activation.* Proc Natl Acad Sci U S A, 2008. **105**(1): p. 64-9.
64. Shimizu, N., et al., *An orphan G protein-coupled receptor, GPR1, acts as a coreceptor to allow replication of human immunodeficiency virus types 1 and 2 in brain-derived cells.* J Virol, 1999. **73**(6): p. 5231-9.
65. Yun, C.W., et al., *Gpr1p, a putative G-protein coupled receptor, regulates glucose-dependent cellular cAMP level in yeast Saccharomyces cerevisiae.* Biochem Biophys Res Commun, 1998. **252**(1): p. 29-33.
66. Uhlenbrock, K., H. Gassenhuber, and E. Kostenis, *Sphingosine 1-phosphate is a ligand of the human gpr3, gpr6 and gpr12 family of constitutively active G protein-coupled receptors.* Cell Signal, 2002. **14**(11): p. 941-53.
67. Bektas, M., et al., *The G protein-coupled receptor GPR4 suppresses ERK activation in a ligand-independent manner.* Biochemistry, 2003. **42**(42): p. 12181-91.
68. Ludwig, M.G., et al., *Proton-sensing G-protein-coupled receptors.* Nature, 2003. **425**(6953): p. 93-8.
69. Zhu, K., et al., *Sphingosylphosphorylcholine and lysophosphatidylcholine are ligands*

for the G protein-coupled receptor GPR4. J Biol Chem, 2001. **276**(44): p. 41325-35.

70. Yin, H., et al., *Lipid G protein-coupled receptor ligand identification using beta-arrestin PathHunter assay.* J Biol Chem, 2009. **284**(18): p. 12328-38.
71. Ignatov, A., et al., *Role of the G-protein-coupled receptor GPR12 as high-affinity receptor for sphingosylphosphorylcholine and its expression and function in brain development.* J Neurosci, 2003. **23**(3): p. 907-14.
72. Lin, Z.J., et al., *GPR12 selections of the metabolites from an endophytic Streptomyces sp. associated with Cistanches deserticola.* Arch Pharm Res, 2008. **31**(9): p. 1108-14.
73. Ciana, P., et al., *The orphan receptor GPR17 identified as a new dual uracil nucleotides/ cysteinyl-leukotrienes receptor.* EMBO J, 2006. **25**(19): p. 4615-27.
74. Kohno, M., et al., *Identification of N-arachidonylglycine as the endogenous ligand for orphan G-protein-coupled receptor GPR18.* Biochem Biophys Res Commun, 2006. **347**(3): p. 827-32.
75. McHugh, D., et al., *Delta(9) -THC and N-arachidonyl glycine are full agonists at GPR18 and cause migration in the human endometrial cell line, HEC-1B.* Br J Pharmacol, 2011.
76. Noguchi, K., S. Ishii, and T. Shimizu, *Identification of p2y9/GPR23 as a novel G protein-coupled receptor for lysophosphatidic acid, structurally distant from the Edg family.* J Biol Chem, 2003. **278**(28): p. 25600-6.
77. Krishnamoorthy, S., et al., *Resolvin D1 binds human phagocytes with evidence for proresolving receptors.* Proc Natl Acad Sci U S A, 2010. **107**(4): p. 1660-5.
78. Sugo, T., et al., *Identification of a lysophosphatidylserine receptor on mast cells.* Biochem Biophys Res Commun, 2006. **341**(4): p. 1078-87.
79. Oka, S., et al., *GPR35 is a novel lysophosphatidic acid receptor.* Biochem Biophys Res Commun, 2010. **395**(2): p. 232-7.
80. Wang, J., et al., *Kynurenic acid as a ligand for orphan G protein-coupled receptor GPR35.* J Biol Chem, 2006. **281**(31): p. 22021-8.
81. Rezgaoui, M., et al., *The neuropeptide head activator is a high-affinity ligand for the orphan G-protein-coupled receptor GPR37.* J Cell Sci, 2006. **119**(Pt 3): p. 542-9.
82. Holst, B., et al., *GPR39 signaling is stimulated by zinc ions but not by obestatin.* Endocrinology, 2007. **148**(1): p. 13-20.
83. Yasuda, S., et al., *Isolation of Zn2+ as an endogenous agonist of GPR39 from fetal bovine serum.* J Recept Signal Transduct Res, 2007. **27**(4): p. 235-46.
84. Johns, D.G., et al., *The novel endocannabinoid receptor GPR55 is activated by atypical cannabinoids but does not mediate their vasodilator effects.* Br J Pharmacol, 2007. **152**(5): p. 825-31.
85. Oka, S., et al., *Identification of GPR55 as a lysophosphatidylinositol receptor.* Biochem Biophys Res Commun, 2007. **362**(4): p. 928-34.
86. Niedernberg, A., et al., *Sphingosine 1-phosphate and dioleoylphosphatidic acid are low affinity agonists for the orphan receptor GPR63.* Cell Signal, 2003. **15**(4): p. 435-46.
87. Im, D.S., et al., *Identification of a molecular target of psychosine and its role in globoid cell formation.* J Cell Biol, 2001. **153**(2): p. 429-34.
88. Wang, J.Q., et al., *TDAG8 is a proton-sensing and psychosine-sensitive G-protein-*

coupled receptor. J Biol Chem, 2004. **279**(44): p. 45626-33.

89. Mogi, C., et al., *Sphingosylphosphorylcholine antagonizes proton-sensing ovarian cancer G-protein-coupled receptor 1 (OGR1)-mediated inositol phosphate production and cAMP accumulation.* J Pharmacol Sci, 2005. **99**(2): p. 160-7.
90. Xu, Y., et al., *Sphingosylphosphorylcholine is a ligand for ovarian cancer G-protein-coupled receptor 1.* Nat Cell Biol, 2000. **2**(5): p. 261-7.
91. Ignatov, A., et al., *RANTES stimulates Ca2+ mobilization and inositol trisphosphate (IP3) formation in cells transfected with G protein-coupled receptor 75.* Br J Pharmacol, 2006. **149**(5): p. 490-7.
92. Wang, J., et al., *Medium-chain fatty acids as ligands for orphan G protein-coupled receptor GPR84.* J Biol Chem, 2006. **281**(45): p. 34457-64.
93. Choi, S., et al., *Identification of a protein hydrolysate responsive G protein-coupled receptor in enterocytes.* Am J Physiol Gastrointest Liver Physiol, 2007. **292**(1): p. G98-G112.
94. Choi, S., et al., *GPR93 activation by protein hydrolysate induces CCK transcription and secretion in STC-1 cells.* Am J Physiol Gastrointest Liver Physiol, 2007. **292**(5): p. G1366-75.
95. Kotarsky, K., et al., *Lysophosphatidic acid binds to and activates GPR92, a G protein-coupled receptor highly expressed in gastrointestinal lymphocytes.* J Pharmacol Exp Ther, 2006. **318**(2): p. 619-28.
96. Lee, C.W., et al., *GPR92 as a new G12/13- and Gq-coupled lysophosphatidic acid receptor that increases cAMP, LPA5.* J Biol Chem, 2006. **281**(33): p. 23589-97.
97. Chu, Z.L., et al., *N-oleoyldopamine enhances glucose homeostasis through the activation of GPR119.* Mol Endocrinol, 2010. **24**(1): p. 161-70.
98. Overton, H.A., et al., *Deorphanization of a G protein-coupled receptor for oleoylethanolamide and its use in the discovery of small-molecule hypophagic agents.* Cell Metab, 2006. **3**(3): p. 167-75.
99. Soga, T., et al., *Lysophosphatidylcholine enhances glucose-dependent insulin secretion via an orphan G-protein-coupled receptor.* Biochem Biophys Res Commun, 2005. **326**(4): p. 744-51.
100. Hirasawa, A., et al., *Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120.* Nat Med, 2005. **11**(1): p. 90-4.
101. Murakami, N., et al., *G2A is a proton-sensing G-protein-coupled receptor antagonized by lysophosphatidylcholine.* J Biol Chem, 2004. **279**(41): p. 42484-91.
102. Obinata, H., et al., *Identification of 9-hydroxyoctadecadienoic acid and other oxidized free fatty acids as ligands of the G protein-coupled receptor G2A.* J Biol Chem, 2005. **280**(49): p. 40676-83.
103. Radu, C.G., et al., *T cell chemotaxis to lysophosphatidylcholine through the G2A receptor.* Proc Natl Acad Sci U S A, 2004. **101**(1): p. 245-50.
104. Dong, X., et al., *A diverse family of GPCRs expressed in specific subsets of nociceptive sensory neurons.* Cell, 2001. **106**(5): p. 619-32.
105. Santos, R.A., et al., *Angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas.* Proc Natl Acad Sci U S A, 2003. **100**(14): p. 8258-63.

106. Shinohara, T., et al., *Identification of a G protein-coupled receptor specifically responsive to beta-alanine.* J Biol Chem, 2004. **279**(22): p. 23559-64.
107. Lembo, P.M., et al., *Proenkephalin A gene products activate a new family of sensory neuron--specific GPCRs.* Nat Neurosci, 2002. **5**(3): p. 201-9.
108. Robas, N., E. Mead, and M. Fidock, *MrgX2 is a high potency cortistatin receptor expressed in dorsal root ganglion.* J Biol Chem, 2003. **278**(45): p. 44400-4.
109. Nakane, Y., et al., *A mammalian neural tissue opsin (Opsin 5) is a deep brain photoreceptor in birds.* Proc Natl Acad Sci U S A, 2010. **107**(34): p. 15264-8.
110. He, W., et al., *Citric acid cycle intermediates as ligands for orphan G-protein-coupled receptors.* Nature, 2004. **429**(6988): p. 188-93.
111. Wellendorph, P., L.D. Johansen, and H. Brauner-Osborne, *Molecular pharmacology of promiscuous seven transmembrane receptors sensing organic nutrients.* Mol Pharmacol, 2009. **76**(3): p. 453-65.
112. Briscoe, C.P., et al., *The orphan G protein-coupled receptor GPR40 is activated by medium and long chain fatty acids.* J Biol Chem, 2003. **278**(13): p. 11303-11.
113. Itoh, Y., et al., *Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40.* Nature, 2003. **422**(6928): p. 173-6.
114. Kotarsky, K., et al., *A human cell surface receptor activated by free fatty acids and thiazolidinedione drugs.* Biochem Biophys Res Commun, 2003. **301**(2): p. 406-10.
115. Nolan, C.J., et al., *Fatty acid signaling in the beta-cell and insulin secretion.* Diabetes, 2006. **55 Suppl 2**: p. S16-23.
116. Brown, A.J., et al., *The Orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids.* J Biol Chem, 2003. **278**(13): p. 11312-9.
117. Le Poul, E., et al., *Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation.* J Biol Chem, 2003. **278**(28): p. 25481-9.
118. Sina, C., et al., *G protein-coupled receptor 43 is essential for neutrophil recruitment during intestinal inflammation.* J Immunol, 2009. **183**(11): p. 7514-22.
119. Sleeth, M.L., et al., *Free fatty acid receptor 2 and nutrient sensing: a proposed role for fibre, fermentable carbohydrates and short-chain fatty acids in appetite regulation.* Nutr Res Rev, 2010. **23**(1): p. 135-45.
120. Ge, H., et al., *Activation of G protein-coupled receptor 43 in adipocytes leads to inhibition of lipolysis and suppression of plasma free fatty acids.* Endocrinology, 2008. **149**(9): p. 4519-26.
121. Xiong, Y., et al., *Short-chain fatty acids stimulate leptin production in adipocytes through the G protein-coupled receptor GPR41.* Proc Natl Acad Sci U S A, 2004. **101**(4): p. 1045-50.
122. Ahmed, K., et al., *Deorphanization of GPR109B as a receptor for the beta-oxidation intermediate 3-OH-octanoic acid and its role in the regulation of lipolysis.* J Biol Chem, 2009. **284**(33): p. 21928-33.
123. Irukayama-Tomobe, Y., et al., *Aromatic D-amino acids act as chemoattractant factors for human leukocytes through a G protein-coupled receptor, GPR109B.* Proc Natl Acad

Sci U S A, 2009. **106**(10): p. 3930-4.
124. Cai, T.Q., et al., *Role of GPR81 in lactate-mediated reduction of adipose lipolysis.* Biochem Biophys Res Commun, 2008. **377**(3): p. 987-91.
125. Liu, C., et al., *Lactate inhibits lipolysis in fat cells through activation of an orphan G-protein-coupled receptor, GPR81.* J Biol Chem, 2009. **284**(5): p. 2811-22.
126. Taggart, A.K., et al., *(D)-beta-Hydroxybutyrate inhibits adipocyte lipolysis via the nicotinic acid receptor PUMA-G.* J Biol Chem, 2005. **280**(29): p. 26649-52.
127. Thangaraju, M., et al., *GPR109A is a G-protein-coupled receptor for the bacterial fermentation product butyrate and functions as a tumor suppressor in colon.* Cancer Res, 2009. **69**(7): p. 2826-32.
128. Ahmed, K., S. Tunaru, and S. Offermanns, *GPR109A, GPR109B and GPR81, a family of hydroxy-carboxylic acid receptors.* Trends Pharmacol Sci, 2009. **30**(11): p. 557-62.
129. Levoye, A. and R. Jockers, *Alternative drug discovery approaches for orphan GPCRs.* Drug Discov Today, 2008. **13**(1-2): p. 52-8.
130. Levoye, A., et al., *Do orphan G-protein-coupled receptors have ligand-independent functions? New insights from receptor heterodimers.* EMBO Rep, 2006. **7**(11): p. 1094-8.
131. Benton, R., et al., *Atypical membrane topology and heteromeric function of Drosophila odorant receptors in vivo.* PLoS Biol, 2006. **4**(2): p. e20.
132. Neuhaus, E.M., et al., *Odorant receptor heterodimerization in the olfactory system of Drosophila melanogaster.* Nat Neurosci, 2005. **8**(1): p. 15-7.
133. White, J.H., et al., *Heterodimerization is required for the formation of a functional GABA(B) receptor.* Nature, 1998. **396**(6712): p. 679-82.
134. Milasta, S., et al., *Interactions between the Mas-related receptors MrgD and MrgE alter signalling and trafficking of MrgD.* Mol Pharmacol, 2006. **69**(2): p. 479-91.
135. Nelson, G., et al., *An amino-acid taste receptor.* Nature, 2002. **416**(6877): p. 199-202.
136. Nelson, G., et al., *Mammalian sweet taste receptors.* Cell, 2001. **106**(3): p. 381-90.
137. Xu, H., et al., *Different functional roles of T1R subunits in the heteromeric taste receptors.* Proc Natl Acad Sci U S A, 2004. **101**(39): p. 14258-63.
138. Levoye, A., et al., *The orphan GPR50 receptor specifically inhibits MT1 melatonin receptor function through heterodimerization.* EMBO J, 2006. **25**(13): p. 3012-23.
139. Riobo, N.A., K. Lu, and C.P. Emerson, Jr., *Hedgehog signal transduction: signal integration and cross talk in development and cancer.* Cell Cycle, 2006. **5**(15): p. 1612-5.
140. Rosenkilde, M.M., et al., *Molecular pharmacological phenotyping of EBI2. An orphan seven-transmembrane receptor with constitutive activity.* J Biol Chem, 2006. **281**(19): p. 13199-208.
141. Rosenkilde, M.M., T.N. Kledal, and T.W. Schwartz, *High constitutive activity of a virus-encoded seven transmembrane receptor in the absence of the conserved DRY motif (Asp-Arg-Tyr) in transmembrane helix 3.* Mol Pharmacol, 2005. **68**(1): p. 11-9.
142. Waldhoer, M., et al., *Murine cytomegalovirus (CMV) M33 and human CMV US28 receptors exhibit similar constitutive signaling activities.* J Virol, 2002. **76**(16): p. 8161-

8.

143. Joost, P. and A. Methner, *Phylogenetic analysis of 277 human G-protein-coupled receptors as a tool for the prediction of orphan receptor ligands.* Genome Biol, 2002. **3**(11): p. RESEARCH0063.

144. Adams, J.W., et al., *Myocardial expression, signaling, and function of GPR22: a protective role for an orphan G protein-coupled receptor.* Am J Physiol Heart Circ Physiol, 2008. **295**(2): p. H509-21.

145. Toyooka, M., T. Tujii, and S. Takeda, *The N-terminal domain of GPR61, an orphan G-protein-coupled receptor, is essential for its constitutive activity.* J Neurosci Res, 2009. **87**(6): p. 1329-33.

146. Jones, P.G., et al., *Tissue distribution and functional analyses of the constitutively active orphan G protein coupled receptors, GPR26 and GPR78.* Biochim Biophys Acta, 2007. **1770**(6): p. 890-901.

147. Hase, M., et al., *Characterization of an orphan G protein-coupled receptor, GPR20, that constitutively activates Gi proteins.* J Biol Chem, 2008. **283**(19): p. 12747-55.

148. Römpler, H., et al., *The rise and fall of the chemoattractant receptor GPR33.* J Biol Chem, 2005. **280**(35): p. 31068-75.

149. Lee, D.K., et al., *Discovery and mapping of ten novel G protein-coupled receptor genes.* Gene, 2001. **275**(1): p. 83-91.

150. Stäubert, C., et al., *Structural and functional evolution of the trace amine-associated receptors TAAR3, TAAR4 and TAAR5 in primates.* PLoS One, 2010. **5**(6): p. e11133.

111. Wellendorph, P., L.D. Johansen, and H. Brauner-Osborne, *Molecular pharmacology of promiscuous seven transmembrane receptors sensing organic nutrients.* Mol Pharmacol, 2009. **76**(3): p. 453-65.

112. Briscoe, C.P., et al., *The orphan G protein-coupled receptor GPR40 is activated by medium and long chain fatty acids.* J Biol Chem, 2003. **278**(13): p. 11303-11.

113. Itoh, Y., et al., *Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40.* Nature, 2003. **422**(6928): p. 173-6.

114. Kotarsky, K., et al., *A human cell surface receptor activated by free fatty acids and thiazolidinedione drugs.* Biochem Biophys Res Commun, 2003. **301**(2): p. 406-10.

115. Nolan, C.J., et al., *Fatty acid signaling in the beta-cell and insulin secretion.* Diabetes, 2006. **55 Suppl 2**: p. S16-23.

116. Brown, A.J., et al., *The Orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids.* J Biol Chem, 2003. **278**(13): p. 11312-9.

117. Le Poul, E., et al., *Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation.* J Biol Chem, 2003. **278**(28): p. 25481-9.

118. Sina, C., et al., *G protein-coupled receptor 43 is essential for neutrophil recruitment during intestinal inflammation.* J Immunol, 2009. **183**(11): p. 7514-22.

119. Sleeth, M.L., et al., *Free fatty acid receptor 2 and nutrient sensing: a proposed role for fibre, fermentable carbohydrates and short-chain fatty acids in appetite regulation.* Nutr Res Rev, 2010. **23**(1): p. 135-45.

120. Ge, H., et al., *Activation of G protein-coupled receptor 43 in adipocytes leads to inhibition of lipolysis and suppression of plasma free fatty acids.* Endocrinology, 2008. **149**(9): p. 4519-26.
121. Xiong, Y., et al., *Short-chain fatty acids stimulate leptin production in adipocytes through the G protein-coupled receptor GPR41.* Proc Natl Acad Sci U S A, 2004. **101**(4): p. 1045-50.
122. Ahmed, K., et al., *Deorphanization of GPR109B as a receptor for the beta-oxidation intermediate 3-OH-octanoic acid and its role in the regulation of lipolysis.* J Biol Chem, 2009. **284**(33): p. 21928-33.
123. Irukayama-Tomobe, Y., et al., *Aromatic D-amino acids act as chemoattractant factors for human leukocytes through a G protein-coupled receptor, GPR109B.* Proc Natl Acad Sci U S A, 2009. **106**(10): p. 3930-4.
124. Cai, T.Q., et al., *Role of GPR81 in lactate-mediated reduction of adipose lipolysis.* Biochem Biophys Res Commun, 2008. **377**(3): p. 987-91.
125. Liu, C., et al., *Lactate inhibits lipolysis in fat cells through activation of an orphan G-protein-coupled receptor, GPR81.* J Biol Chem, 2009. **284**(5): p. 2811-22.
126. Taggart, A.K., et al., *(D)-beta-Hydroxybutyrate inhibits adipocyte lipolysis via the nicotinic acid receptor PUMA-G.* J Biol Chem, 2005. **280**(29): p. 26649-52.
127. Thangaraju, M., et al., *GPR109A is a G-protein-coupled receptor for the bacterial fermentation product butyrate and functions as a tumor suppressor in colon.* Cancer Res, 2009. **69**(7): p. 2826-32.
128. Ahmed, K., S. Tunaru, and S. Offermanns, *GPR109A, GPR109B and GPR81, a family of hydroxy-carboxylic acid receptors.* Trends Pharmacol Sci, 2009. **30**(11): p. 557-62.
129. Levoye, A. and R. Jockers, *Alternative drug discovery approaches for orphan GPCRs.* Drug Discov Today, 2008. **13**(1-2): p. 52-8.
130. Levoye, A., et al., *Do orphan G-protein-coupled receptors have ligand-independent functions? New insights from receptor heterodimers.* EMBO Rep, 2006. **7**(11): p. 1094-8.
131. Benton, R., et al., *Atypical membrane topology and heteromeric function of Drosophila odorant receptors in vivo.* PLoS Biol, 2006. **4**(2): p. e20.
132. Neuhaus, E.M., et al., *Odorant receptor heterodimerization in the olfactory system of Drosophila melanogaster.* Nat Neurosci, 2005. **8**(1): p. 15-7.
133. White, J.H., et al., *Heterodimerization is required for the formation of a functional GABA(B) receptor.* Nature, 1998. **396**(6712): p. 679-82.
134. Milasta, S., et al., *Interactions between the Mas-related receptors MrgD and MrgE alter signalling and trafficking of MrgD.* Mol Pharmacol, 2006. **69**(2): p. 479-91.
135. Nelson, G., et al., *An amino-acid taste receptor.* Nature, 2002. **416**(6877): p. 199-202.
136. Nelson, G., et al., *Mammalian sweet taste receptors.* Cell, 2001. **106**(3): p. 381-90.
137. Xu, H., et al., *Different functional roles of T1R subunits in the heteromeric taste receptors.* Proc Natl Acad Sci U S A, 2004. **101**(39): p. 14258-63.
138. Levoye, A., et al., *The orphan GPR50 receptor specifically inhibits MT1 melatonin receptor function through heterodimerization.* EMBO J, 2006. **25**(13): p. 3012-23.

139. Riobo, N.A., K. Lu, and C.P. Emerson, Jr., *Hedgehog signal transduction: signal integration and cross talk in development and cancer.* Cell Cycle, 2006. **5**(15): p. 1612-5.
140. Rosenkilde, M.M., et al., *Molecular pharmacological phenotyping of EBI2. An orphan seven-transmembrane receptor with constitutive activity.* J Biol Chem, 2006. **281**(19): p. 13199-208.
141. Rosenkilde, M.M., T.N. Kledal, and T.W. Schwartz, *High constitutive activity of a virus-encoded seven transmembrane receptor in the absence of the conserved DRY motif (Asp-Arg-Tyr) in transmembrane helix 3.* Mol Pharmacol, 2005. **68**(1): p. 11-9.
142. Waldhoer, M., et al., *Murine cytomegalovirus (CMV) M33 and human CMV US28 receptors exhibit similar constitutive signaling activities.* J Virol, 2002. **76**(16): p. 8161-8.
143. Joost, P. and A. Methner, *Phylogenetic analysis of 277 human G-protein-coupled receptors as a tool for the prediction of orphan receptor ligands.* Genome Biol, 2002. **3**(11): p. RESEARCH0063.
144. Adams, J.W., et al., *Myocardial expression, signaling, and function of GPR22: a protective role for an orphan G protein-coupled receptor.* Am J Physiol Heart Circ Physiol, 2008. **295**(2): p. H509-21.
145. Toyooka, M., T. Tujii, and S. Takeda, *The N-terminal domain of GPR61, an orphan G-protein-coupled receptor, is essential for its constitutive activity.* J Neurosci Res, 2009. **87**(6): p. 1329-33.
146. Jones, P.G., et al., *Tissue distribution and functional analyses of the constitutively active orphan G protein coupled receptors, GPR26 and GPR78.* Biochim Biophys Acta, 2007. **1770**(6): p. 890-901.
147. Hase, M., et al., *Characterization of an orphan G protein-coupled receptor, GPR20, that constitutively activates Gi proteins.* J Biol Chem, 2008. **283**(19): p. 12747-55.
148. Römpler, H., et al., *The rise and fall of the chemoattractant receptor GPR33.* J Biol Chem, 2005. **280**(35): p. 31068-75.
149. Lee, D.K., et al., *Discovery and mapping of ten novel G protein-coupled receptor genes.* Gene, 2001. **275**(1): p. 83-91.
150. Stäubert, C., et al., *Structural and functional evolution of the trace amine-associated receptors TAAR3, TAAR4 and TAAR5 in primates.* PLoS One, 2010. **5**(6): p. e11133.