

## REVIEW

# Orphan GPCR research

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Orphan G protein-coupled receptors (GPCRs) are receptors lacking endogenous ligands. Found by molecular biological analyses, they became the roots of reverse pharmacology, in which receptors are attempted to be matched to potential transmitters. Later, when high-throughput screening technology was applied to reverse pharmacology, dozens of orphan GPCRs became deorphanized. Furthermore, novel neuropeptides were discovered. This review retraces the history of the orphan GPCRs and of the discoveries of their endogenous ligands, it also discusses the difficulties that the search for new ligands is presently encountering.

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**Abbreviations:** AGRP, agouti-related protein; GPCRs, G protein-coupled receptors; MCH, melanin-concentrating hormone; NPY, neuropeptide Y

## Introduction

Most G protein-coupled receptors (GPCRs) were found by sequence similarity. These, however, lack endogenous ligands and thus are orphan GPCRs. Although molecular biological and bioinformatics techniques made the identification of orphan GPCRs amenable, the search for their endogenous ligands has been a challenge. This search has given birth to the reverse pharmacology approach, which uses orphan GPCRs as targets to identify endogenous ligands. This approach was very successful and has led over two decades to the deorphanization of about 300 GPCRs. Many of the ligands that were matched to orphan GPCRs were already known, but a dozen are novel and in particular, nine novel neuropeptide families were discovered. These novel neuropeptides have already enriched our understanding of several important behavioural responses, in particular, the central regulation of food intake. Presently, there exist still 100 orphan GPCRs that may bind endogenous ligands that are not chemosensory. This review recounts the history of orphan GPCR research, discusses some of its successes and its present difficulties.

## History of the orphan GPCR research

Orphan GPCRs are receptors lacking endogenous ligands. Their history began with the discovery that the  $\beta_2$ -adrenergic

receptor and the opsins share a seven-transmembrane domain topology (Dixon *et al.*, 1986). These two receptors were not known to have much in common except that both couple to G proteins to elicit intracellular responses. This gave rise to the concept that all the receptors that couple to G proteins may form a supergene family, thereafter called the GPCRs. This concept gained rapid credence when metabotropic receptors for other neurotransmitters such as acetylcholine and serotonin were shown to be GPCRs (Hall, 1987). It ascertained itself when the receptor for a neuropeptide, substance K, was also found to be a GPCR (Masu *et al.*, 1987).

## The discovery of the orphan GPCRs

The GPCR concept had an immediate consequence—nucleic acid-based homology screening approaches could be applied to find new GPCRs. Low-stringency hybridization (Bunzow *et al.*, 1988) and PCR-derived approaches (Libert *et al.*, 1989) were applied in the second part of the 1980s and paved the way for the discoveries of many GPCRs. This was boosted one step higher, 10 years later with the sequence of the genome, when bioinformatic analyses put the number of human GPCRs to about 800 (Vassilatis *et al.*, 2003).

The discovery of new GPCRs found by homology screening suffers from one obvious problem; these receptors lack their pharmacological identities, that is their endogenous ligands. They are all 'orphan' receptors (Libert *et al.*, 1991). As the vast majority of them are conserved in different species, one could assume that they are functionally active. However, the

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search for their endogenous ligands seemed to be a formidable, and possibly irresolvable task.

From what had been learned from GPCR cloning in 1986–1987, one could infer that probably all the small molecules that direct intercellular interactions would interact with GPCRs. These are the first messengers, the ‘transmitters’, that are released from a cell to carry a message to a second one. They act in endocrine, paracrine or exocrine fashion to allow the organism to react to particular physiological challenges. These transmitters are mostly small molecules although few of them are larger polypeptides. They include biogenic amines, neuropeptides, lipid mediators, nucleotides, amino acid and derivatives, polypeptide hormones and, as will be learned later, chemokines, olfactory and gustatory molecules and some other naturally occurring chemicals such as calcium ions and protons. How could one find which one of these transmitters may bind an orphan GPCR?

#### *The deorphanization of GPCRs*

The strategy was to express the orphan GPCR of interest in eukaryotic cells by DNA transfection and to use membranes of these cells as targets for testing the binding abilities of potential ligands. This strategy is now known as reverse pharmacology (Libert *et al.*, 1991; Mills and Duggan, 1994). At the end of the 1980s, there existed about 50 transmitters (in particular, many neuropeptides) that were potential GPCR ligands and had no cognate receptors. The reverse pharmacology approach meant carrying numerous binding assays using a plethora of radioactive ligands, an impossible task in an academic environment. Yet, serendipity and ingenious insights proved to be successful in matching the first orphan GPCRs.

In 1987, G-21 was the first orphan GPCR reported through its sequence similarities to the  $\beta_2$ -adrenergic receptors. Attempts at binding adrenergic ligands to G-21 proved unsuccessful, until it was recognized that it binds serotonergic ligands. G-21 is now known as the 5-HT<sub>1A</sub> receptor (Fargin *et al.*, 1988). We also screened a rat genomic library with a  $\beta_2$ -adrenergic probe at low stringency and found numerous hybridizing clones. One in particular, RGB-2, was interesting as it had the seven-transmembrane domain hallmark of the GPCRs, yet was encoded by a gene containing introns and thus evolutionary different from the  $\beta_2$ -adrenergic receptors. Moreover, RGB-2 was not only expressed in the CNS but also, at high levels, in the anterior and intermediate lobes of the pituitary. This led us to hypothesize that it may be the dopamine D<sub>2</sub> receptor that was then rapidly demonstrated in specific binding assays (Bunzow *et al.*, 1988). It is worth noting that some of the other clones that were found using the low-stringency screening approach led us to report the sequences of the rat  $\beta_1$ -adrenergic and 5-HT<sub>1A</sub> receptors (Albert *et al.*, 1990; Machida *et al.*, 1990).

In 1989, the group of Parmentier and Vassart reported the use of the, at that time somewhat new, PCR technology as a way of finding new GPCRs (Libert *et al.*, 1989). They had recognized that many of the cloned GPCRs contained conserved sequences in their third and sixth transmembrane domains, which allowed for designing DNA probes that

could serve as general primers under non-stringent hybridization conditions. Applying this approach, they were able to identify half a dozen new orphan GPCRs. Because of its speed, the PCR-based approach had an important impact on the search for orphan GPCRs (Civelli *et al.*, 2006). It became the method of choice for finding new orphan GPCRs, whereas low-stringency screening remained the most used approach for finding subtypes of cloned GPCRs.

#### *The search for novel transmitters*

During the first part of the 90s, application of the reverse pharmacology strategy led to the pharmacological characterization of many GPCRs. Thus, the number of potential orphan GPCR ligands was decreasing. At the same time, random searches for new GPCRs, using PCR-based homology screening approaches were in full swing, thus the overall number of orphan GPCRs was steadily increasing (Marchese *et al.*, 1994, 1999). This led us to conclude that many orphan GPCRs must bind undiscovered ligands.

This conclusion was based on the fact that the vast majority of orphan GPCRs are evolutionarily conserved and thus are expected to be active. Although the seven-transmembrane domain topology has since evolved to include receptors that do not couple to G proteins, and although our understanding of receptor activation has been shown to be complex to include proteins other than the G proteins (Bockaert and Pin, 1999; Angers *et al.*, 2002; Pierce *et al.*, 2002; Kristiansen, 2004), at that time, it was expected that an activated orphan GPCR would couple to a G protein and induce a second messenger response. Consequently, monitoring second messenger levels induced by an orphan GPCR should lead to the identification of its ligand. As there was evidence that GPCRs can be promiscuous and activate more than one second messenger response especially when expressed at high levels as occurs upon heterologous transfection, one did not need to know the natural transduction pathway of an orphan GPCR *a priori*. Furthermore, by the mid-1990s, assays had been developed that allowed for high-throughput detection of second messenger responses. So reverse pharmacological approaches based on receptor reactivity instead of receptor binding could be applied.

The source of ligands of orphan GPCRs was another issue. From a basic stand point, isolating the natural ligand of an orphan GPCR was seen as most rewarding as it would lead to the identification of a novel transmitter and thus open new doors in our understanding of physiology. Most orphan GPCRs are expressed in the brain indicating that brain extracts would be good starting materials. As purification techniques have been developed that identify small amounts of peptides and as many orphan GPCRs bare the similarities of known neuropeptide GPCRs, the first attempts were directed at identifying novel neuropeptides.

#### **The novel neuropeptides**

The first orphan GPCR that was used for discovering a novel transmitter was opioid receptor-like 1, cloned through its

homology to the opioid receptors (Henderson and McKnight, 1997). Its activation was monitored by quantifying intracellular decreases in cAMP levels, which could be measured in newly developed scintillation proximity assays. Because its phylogenetic analyses classified opioid receptor-like 1 as a peptidergic GPCR and because opioid receptor-like 1 is expressed in the CNS, peptidergic brain tissue extracts were prepared, purified and fractionated. Fractions were tested for their abilities to inhibit AC activity in cells that were stably transfected with opioid receptor-like 1. A 17-residue long peptide was ultimately isolated, named orphanin FQ or nociceptin (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995). Its structural similarities to the opioid peptides made it an immediate star, yet it has been proven not to bind the opioid receptors (Reinscheid *et al.*, 1998).

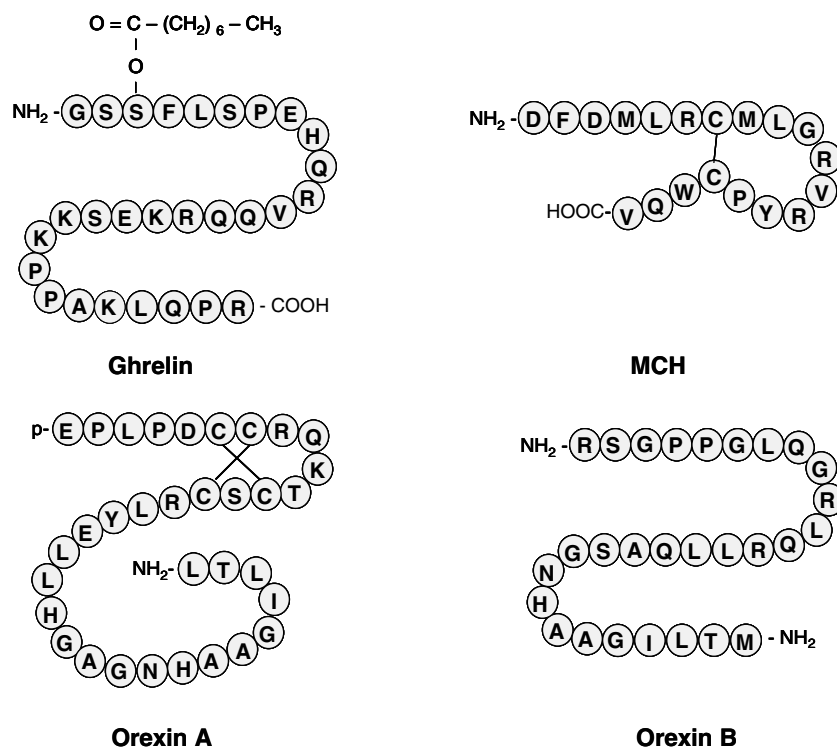
The second successful attempt at discovering novel neuropeptides through orphan GPCRs screened over 50 different orphan GPCRs by measuring their abilities to induce intracellular calcium release when subjected to peptidic extracts. One receptor did respond and led to the characterization of two peptides, the orexins (Sakurai *et al.*, 1998), also identified through an RNA subtraction approach as hypocretins (de Lecea *et al.*, 1998). This was immediately followed by the discovery of prolactin-releasing peptide and apelin as the natural ligands of the orphan GPCRs, GPR10 and putative receptor protein related to the angiotensin receptor (AT<sub>1</sub>), respectively (Hinuma *et al.*, 1998; Tatemoto *et al.*, 1998). Since then, the novel neuropeptides ghrelin, metastin, neuropeptides B/W, prokineticins1/2, neuropeptide S and neuromedin S have been discovered as natural ligands of orphan GPCRs (Civelli *et al.*, 2006).

These successes proved not only the validity of the orphan GPCR approach for finding novel transmitters but also the impact of high-throughput screening of orphan GPCRs. It is therefore not surprising that the pharmaceutical industry became its major proponent (Hinuma *et al.*, 1999). Consequently, orphan GPCRs began to be screened randomly against large libraries of synthetic ligands (Wise *et al.*, 2004). These libraries contained all the ligands that had not been matched to any receptor molecules but also many molecules that are known to exist in the cells. This led in a few years to the deorphanization of some 40 GPCRs (Saito and Civelli, 2005).

### Impact of the orphan GPCR research

As described above, the search for new GPCRs by homology screening coincides with the start of the orphan GPCR research. It is this search that led to the discovery of practically all the GPCRs that are studied today. But it is the deorphanization of GPCRs that are of importance and continue to be of importance because it impacts our understanding of the organism's function.

The most striking examples came from the discoveries of the novel neuropeptides. These neuropeptides have been implicated in an increasing number of physiological responses such as stress, sleep and circadian rhythm. Three of these neuropeptides, in particular, have had a direct impact on our understanding of the central regulation of food intake (Figure 1).



**Figure 1** Amino-acid sequences of human ghrelin, MCH, orexin A and orexin B. The third residue of ghrelin, serine, is *n*-octanoylated. MCH possesses an intrachain disulphide bond. Orexin A possesses an N-terminal pyroglutamyl residue and two intrachain disulphide bonds. Both orexins are C-terminally amidated. MCH, melanin-concentrating hormone.

## Impact on nutritional homoeostasis

### *The orexin system*

Orexins were identified as endogenous ligands for orphan GPCRs (Sakurai *et al.*, 1998), although they were also discovered by using mRNA subtraction strategy (de Lecea *et al.*, 1998). The orexins (orexin A and B) activate two related GPCRs, but are encoded by one precursor peptide, prepro-orexin, which is exclusively synthesized in the lateral hypothalamus. This pattern of expression indicated that the orexins may be regulators of feeding behaviour. Indeed it was found that fasting upregulated prepro-orexin mRNA gene and that central administration of orexins increases spontaneous food intake (Sakurai *et al.*, 1998). Orexin-expressing neurons are also activated by peripheral metabolic signals, such as leptin, ghrelin and glucose, indicating that these neurons integrate energy-related signals coming from the periphery to the brain (Yamanaka *et al.*, 2003). Consistent with these data, mice lacking prepro-orexin or mice in which orexin neurons have been ablated exhibit a hypophagic phenotype (Willie *et al.*, 2001). Yet, mice lacking orexin neurons develop late-onset obesity in spite of being hypophagic, which indicates that the orexin system regulates energy expenditure as well.

It is noteworthy that orexin deficiency also caused a narcoleptic phenotype in humans and animals, indicating a role of the orexin system in the maintenance of wakefulness (Chemelli *et al.*, 1999; Lin *et al.*, 1999; Peyron *et al.*, 2000; Thannickal *et al.*, 2000). These two characteristics, increase feeding and maintenance of wakefulness, point at the orexin system as being important in arousal during food searching. Maintenance of arousal during food searching is important for survival. Daily restricted feeding induces an anticipatory locomotor activity. Consequently, it was shown that the activity of orexin neurons is markedly increased during the food-anticipatory period under restricted feeding (Akiyama *et al.*, 2004; Mieda *et al.*, 2004). Furthermore, mice lacking orexin neurons display, under restricted feeding, reduced food-anticipatory increases in wakefulness and locomotor activity (Akiyama *et al.*, 2004). These observations indicate that the orexin system is important in conveying the information about the metabolic status to increase wakefulness and search for food. The orexin system thus provides a link between energy homoeostasis and wakefulness.

### *The MCH system*

Melanin-concentrating hormone (MCH) had been known for 20 years as a peptide that induces paling of the skin in fish (Kawauchi *et al.*, 1983) and since the mid-1990s, as a hypothalamic neuropeptide that is upregulated by fasting in rats (Qu *et al.*, 1996). However, the full impact of the MCH system could not be studied until an orphan GPCR, SLC-1, was shown to be the MCH receptor (Bachner *et al.*, 1999; Chambers *et al.*, 1999; Lembo *et al.*, 1999; Saito *et al.*, 1999; Shimomura *et al.*, 1999).

Acute central administrations of MCH increase food intake whereas chronic injections increase body weight, white adipose tissue mass and liver mass (Qu *et al.*, 1996; Della-Zuana *et al.*, 2002). Mice lacking MCH are lean due to

hypophagia and an increase in metabolic rate (Shimada *et al.*, 1998). MCH receptor knockout mice are also lean, yet they are hyperactive and hyperphagic (Marsh *et al.*, 2002). To understand if MCH contributes to the hyperphagic phenotype of ob/ob mice, double-null mice lacking both MCH and leptin were generated (Segal-Lieberman *et al.*, 2003). Surprisingly, these mice are still hyperphagic but present less body fat suggesting that the MCH system is critical for regulating energy expenditure and not for the maintenance of the hyperphagic phenotype in ob/ob mice. MCH may regulate energy expenditure through the hindbrain (Zheng *et al.*, 2005). MCH administration into the fourth ventricle with the intent to target the nucleus tractus solitarius decreases body temperature without changing physical activity and feeding. This indicates that MCH has an inhibitory role in energy expenditure through hindbrain region (Zheng *et al.*, 2005).

Although the MCH<sub>1</sub> receptor is predominantly expressed in the brain, significant levels are also found in peripheral organs such as the pancreas, the skeletal muscle, the eye and the fat cells (Saito *et al.*, 1999; Hill *et al.*, 2001). The MCH<sub>1</sub> receptor is expressed in insulin-producing cells and in the islets of Langerhans, where MCH significantly stimulates insulin secretion. This insulinotropic effect may contribute to the regulation of metabolism and energy balance (Tadayyon *et al.*, 2000). The MCH<sub>1</sub> receptor is also present in rat adipocytes and stimulates leptin production (Bradley *et al.*, 2000). Together, these data show that the MCH system, although prominently viewed as a central system regulating energy homoeostasis, also has peripheral actions that impact on leptin or insulin release.

### *The ghrelin system*

Ghrelin, a 28-amino-acid peptide with an *n*-octanoylation, was isolated as the endogenous ligand for the growth hormone secretagogue (GHS) receptor (Kojima *et al.*, 1999). Growth hormone secretagogues are small synthetic molecules that act on the pituitary gland and the hypothalamus to stimulate growth hormone release (Howard *et al.*, 1996; McKee *et al.*, 1997).

Ghrelin is synthesized in the stomach. Peripheral and central administrations of ghrelin were shown to stimulate GH secretion and increase food intake and body weight (Wren *et al.*, 2000, 2001). In rats, fasting increases plasma ghrelin levels, whereas feeding decreases them (Wren *et al.*, 2001). In humans, plasma ghrelin levels rise before a meal and fall after the meal (Cummings *et al.*, 2001). Ghrelin is therefore a regulator of energy homoeostasis, which carries its message from the periphery to the CNS. It is worth noting that ghrelin is synthesized as part of a larger precursor that has been reported to encode another bioactive peptide obestatin (Zhang *et al.*, 2005). Obestatin was heralded as having the opposite effects of ghrelin, yet its role and even existence is doubtful (Lauwers *et al.*, 2006; Seoane *et al.*, 2006; Yamamoto *et al.*, 2007).

Systemic administration of ghrelin activates Fos expression in the arcuate nucleus in the brain pointing to this nucleus as the site of circulating ghrelin action (Hewson and Dickson, 2000). Central administration of ghrelin activates

Fos immunoreactivity in several brain regions, primarily in regions implicated in the regulation of feeding behaviour, including neuropeptide Y (NPY)/agouti-related protein (AGRP)-expressing neurons in the arcuate nucleus (Nakazato *et al.*, 2001). The arcuate nucleus is a crucial target of leptin action. Leptin is the satiety signal that is released from adipocytes upon feeding and reaches the hypothalamus where it inhibits the activity of NPY/AGRP neurons (Stephens *et al.*, 1995; Schwartz *et al.*, 1996). Concordantly, it was found that leptin inhibits the orexigenic effects of ghrelin whereas ghrelin reverses the anorexic effect of leptin (Nakazato *et al.*, 2001). However, although ghrelin is thought to be able to cross the blood–brain barrier to act on the brain, peripheral injections of ghrelin to vagotomized mice neither stimulated feeding nor activated NPY/AGRP neurons (Date *et al.*, 2002). This on the other hand suggests that the pathway relaying ghrelin's signals to the brain may not be through the arcuate nucleus but via the gastric vagal afferents.

The discovery of the ghrelin system revealed how appetite-regulating factor secreted from peripheral organs can control energy homeostasis via the CNS. In contrast to leptin, which is secreted from adipocytes as an appetite suppressing signal for the brain, ghrelin is secreted from the stomach as a hunger signal for the brain. Unlike the most orexigenic neuropeptides found in hypothalamus, such as NPY, AGRP, orexin, MCH or galanin, ghrelin is the first circulating neuropeptide that enhances feeding following systemic administration.

## Impact on drug discovery

The matching of orphan GPCRs to previously known transmitters did not lead to the discovery of novel signalling molecules. Yet it has opened the opportunity to combine anatomical studies on the site of synthesis of the transmitters with that on their sites of action and thus to gain a full

understanding of the localization of the system. Most often, the sites of the orphan GPCR expression serve as primary indication of the role of the system. Genetic ablations of the orphan GPCRs have also helped to understand these new receptor systems. Moreover, the matching of orphan GPCRs to known transmitters has had a major impact on drug discovery.

G protein-coupled receptors are one of the most important drug targets for the pharmaceutical industry as evidenced by the fact that approximately 30% of all marketed drugs act on them (Hopkins and Groom, 2002). The deorphanization successes allowed the pharmaceutical industry to initiate drug screening projects directed at the respective receptors. That is to say, transmitter systems that previously could not be targeted in drug discovery became instantaneously amenable to compound screening. This had and continues to have a profound impact on drug development. Many deorphanized GPCR systems have entered drug design programs, in particular, directed at the three deorphanized systems discussed above. Numerous MCH<sub>1</sub> receptor antagonists have been reported and several lines of preclinical studies have indicated that blockade of the MCH<sub>1</sub> receptor serves as a promising pharmacological target for the treatment of obesity as well as depression and anxiety (McBriar, 2006; Shimazaki *et al.*, 2006). Indeed, currently, an MCH<sub>1</sub> receptor antagonist (NGD-4715; Neurogen Corp., Branford, CT, USA) is undergoing clinical trials. Furthermore, ghrelin (GTP-200; Gastrotech Pharma A/S, Copenhagen K, Denmark) and orexin receptor antagonists (ACT-078573; Actelion Pharmaceuticals Ltd, GSK649868; GlaxoSmithKline plc., Brentford, UK) are also potential drugs and are currently in phase II clinical trials for the treatment of catabolic and sleep disorders, respectively.

So there is no doubt that deorphanized GPCR systems will be future therapeutic targets. In Table 1, we describe a list of deorphanized GPCRs that are in pharmaceutical development, although this evidently may not include all of them. Recognizing the important effort that the pharmaceutical industry has made in espousing the deorphanization of

**Table 1** Deorphanized GPCRs reported to be in drug discovery

Orphan receptor	Ligand	Therapeutic indication	Action of the compounds	References
ORL-1 (NOP) Edg1, 3, 5, 6, 8 H3	Nociceptin/Orphanin FQ S1P Histamine	Stress and pain Autoimmune diseases Dementia	Agonist Agonist/antagonist Antagonist/inverse agonist	Shoblock (2007) Chiba (2005); Foss <i>et al.</i> (2007) Hancock (2006)
Orexin1, 2 SLC-1 (MCH1)	OrexinA and B MCH	Sleep disorders Obesity, anxiety and depression	Antagonist Antagonist	Bingham <i>et al.</i> (2006) McBriar (2006); Shimazaki <i>et al.</i> (2006)
GHSR GPR38	Ghrelin Motilin	Catabolic disorders Gastroparesis and irritable bowel syndrome	Agonist Agonist	Davenport <i>et al.</i> (2005) McCallum <i>et al.</i> (2007)
GPRv53 (H4) P2Y12 GPR16 (BLT1)	Histamine ADP LTB4	Inflammation Platelet aggregation Inflammation and rheumatoid arthritis	Antagonist Antagonist Antagonist	Dunford <i>et al.</i> (2007) van Giezen and Humphries (2005) Ding <i>et al.</i> (2005); Lundeen <i>et al.</i> (2006)
BLT2	LTB4	Inflammation and rheumatoid arthritis	Antagonist	Ding <i>et al.</i> (2005); Lundeen <i>et al.</i> (2006)
HG55 (CysLT1) GPR40	LTD4 Medium and long fatty acids	Bronchoconstriction Diabetes	Antagonist Agonist	Nayak (2004) Song <i>et al.</i> (2007)
HM74A, B	Nicotinic acid	Dyslipidaemia	Agonist	Offermanns (2006)

GPCRs, we can expect that deorphanized GPCR systems will be at the origin of several of the drugs in the future.

### The future of orphan GPCR research

The number of orphan GPCRs is still large. Of the ~800 GPCRs, some 500 are chemosensory; they include the chemokines and chemoattractant GPCRs, which have mostly been deorphanized and the olfactory and gustatory GPCRs, which in large part remain orphans. The transmitter GPCRs, the focus of this review, account for about 360 GPCRs of which 100 are still orphans. The rate at which transmitter GPCRs were deorphanized at the turn of the century has drastically decreased. At that time, some 10 GPCRs were deorphanized per year, whereas very few have been deorphanized since 2004. Moreover, no novel transmitter has been discovered since then. This raises the question of whether orphan GPCR research has reached the end of its road and, if so, what its causes are. There are several issues that account for this.

The first issue concerns the fact that we have depleted the pool of known transmitters. Practically, all the possible transmitters have been matched to GPCRs and, consequently, the remaining orphan GPCRs can only bind unknown transmitters. Finding these as described above is a risky, lengthy and demanding endeavour that cannot be undertaken if immediate results are desired. It is thus not surprising that many have abandoned this route. One had also hoped that the sequencing of the human genome may reveal candidate bioactive peptides. But thus far, only the family of the QRFP peptides has been found using a bioinformatic approach. (Fukusumi *et al.*, 2003). In this respect, it should also be mentioned that one expects that only 20% of the remaining orphan GPCRs will be neuropeptide receptors, whereas the rest will bind nucleotides, lipid mediators and neurotransmitters. Thus bioinformatic approaches can only have a limited impact on the deorphanization of the still orphan GPCRs.

The second issue relies on the finding that GPCRs exist not as monomers but as dimers or higher oligomers (Bulenger *et al.*, 2005). It is now accepted that many GPCRs associate not only to themselves but also to other GPCRs to activate their signalling cascade. Heteromers carry a different pharmacological profile than the monomers. It is thus possible that some of the remaining orphan GPCRs do not act alone but in conjunction with other GPCRs. They would not induce their own second messenger pathway but would modulate that of other GPCRs. Indeed, such a possibility has been described in the case of a melatonin-like orphan GPCR, which has been shown to act as a negative modulator of the melatonin 1 receptor (Levoye *et al.*, 2006). If orphan GPCRs heterodimerize with other GPCRs, finding their role becomes an about impossible task as they may associate with any of the 260 deorphanized GPCRs. Related to this issue is also the possibility that some orphan GPCRs may require the expression of accessory proteins for their activity. This has been shown in the case of the calcitonin GPCR, which necessitate the presence of RAMPs for inducing its signalling pathway (McLatchie *et al.*, 1998; Hay *et al.*, 2006).

The third issue concerns the fact that presently, the reverse pharmacological approach relies on monitoring changes in second messenger levels and thus a knowledge of that pathway. Although expect that GPCRs induce second messenger responses via G proteins, there are indications that some may link to different transducing pathways, some of which may not have been found. If this was the case, the deorphanization of the remaining GPCRs will await until these pathways have been defined.

The fourth issue concerns the concentrations of the transmitters in their natural environment. It is possible that some transmitters are only expressed at a particular time in the life of the organism or under particular conditions. If this is the case, then knowledge of those transmitter biology will be required before matching to their orphan GPCRs can be undertaken.

But, in spite of these caveats, it remains that the search for the ligands of orphan GPCRs will continue to serve as an important approach in discovering novel transmitters. Any one of these opens the door for a different understanding of particular physiological responses and for uncharted therapeutic developments.

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### Conflict of interest

The authors state no conflict of interest.

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