Do orphan G-protein-coupled receptors have ligand-independent functions?

New insights from receptor heterodimers

Angélique Levoye^{1-4*}, Julie Dam^{1-4*}, Mohammed A. Ayoub^{1-4†}, Jean-Luc Guillaume¹⁻⁴ & Ralf Jockers¹⁻⁴⁺

¹Institut Cochin, ²Inserm, U567, ³CNRS, UMR 8104, and ⁴Université Paris Descartes, UMR-S 8104, Paris, France

G-protein-coupled receptors (GPCRs) are important drug targets and are involved in virtually every biological process. However, there are still more than 140 orphan GPCRs, and deciphering their function remains a priority for fundamental and clinical research. Research on orphan GPCRs has concentrated mainly on the identification of their natural ligands, whereas recent data suggest additional ligand-independent functions for these receptors. This emerging concept is connected with the observation that orphan GPCRs can heterodimerize with GPCRs that have identified ligands, and by so doing regulate the function of the latter. Pairing orphan GPCRs with their potential heterodimerization partners will have a major impact on our understanding of the extraordinary diversity offered by GPCR heterodimerization and, in addition, will constitute a novel strategy to elucidate the function of orphan receptors that needs to be added to the repertoire of 'deorphanization' strategies.

Keywords: GPCR; melatonin; melatonin-related receptor; orphan receptors; heterodimerization; GPR50

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Introduction

The superfamily of G-protein-coupled receptors (GPCRs) is one of the largest and most studied families of proteins. Its members respond to an extensive panel of diverse ligands and participate in an extraordinary number of physiological functions. It has now become widely accepted that many, if not all, of the approximately 400 non-odorant GPCRs exist as homo- and heterodimers, which has important functional consequences for receptor pharmacology, signalling and regulation (Milligan, 2004; Prinster *et al*, 2005;

Terrillon & Bouvier, 2004). So far, mainly GPCRs with known ligands and functions have been studied, but recent findings clearly show that the idea of GPCR dimerization can also be extended to orphan (without a known ligand) GPCRs. Despite the vast and long-standing efforts of academic and industrial research to pair these receptors to potential ligands, more than 140 of the non-odorant GPCRs remain orphans (Civelli, 2005). Standard deorphanization strategies seem to have reached their limit and new strategies are urgently required. In this review, we focus our attention on the heterodimerization of orphan and non-orphan GPCRs. This type of association implies that orphan GPCRs might have ligand-independent properties, which provides a novel concept for the elucidation of the function of orphan GPCRs. To distinguish these 'real' orphans from receptors that are probably regulated by as yet unidentified ligands, we use the term orphan seven-transmembrane (7TM) protein in this review. Known examples supporting this concept are presented (Table 1), and the consequences of ligand-independent functions of orphan 7TM proteins are discussed.

Regulation of receptor export to the cell surface

The fact that GPCR dimers are formed early in the biosynthetic pathway led to the proposal that GPCR dimerization promotes receptor folding, maturation and/or transport to the cell surface (Bulenger *et al*, 2005). The metabotropic γ -aminobutyric acid B (GABA_B) receptor has been instrumental in establishing this idea. A functional GABA_B receptor that can be isolated from brain tissue is a heterodimer composed of two homologous subunits called GABA_{B1} and GABA_{B2} (Jones *et al*, 1998; Kaupmann *et al*, 1998; White *et al*, 1998). Interestingly, evolution preferentially selected a system in which each subunit of this obligatory heterodimer has a distinct function: GABA_{B1} provides ligand binding, whereas GABA_{B2} promotes efficient transport of GABA_{B1} to the cell surface and G-protein coupling (Pin *et al*, 2005). An intracellular endoplasmic reticulum retention signal on GABA_{B1}, which prevents GABA_{B1} from reaching the cell surface by itself, is masked in the GABA_{B1}/GABA_{B2} heterodimer. Evolutionary trace analysis showed that the putative GABA binding site of GABA_{B2} is under no evolutionary pressure, demonstrating that this binding site is indeed not functional. Thus, GABA_{B2} can be considered to be an orphan 7TM protein in the heterodimer (Kniazeff *et al*, 2002).

¹Institut Cochin, Department of Cell Biology, ²Inserm, U567, ³CNRS, UMR 8104, and ⁴Université Paris Descartes, Faculté de Médecine René Descartes, UMR-S 8104, 22 rue Méchain, Paris F-75014, France

[†]Present address: Institut de Génétique Fonctionnelle (IGF), UMR5203 CNRS, U661 INSERM, Universités Montpellier 1 & 2, Département de Pharmacologie Moléculaire, 141 rue de la Cardonille 34094, Montpellier Cedex 05, France

*These authors contributed equally to this work

⁺Corresponding author. Tel: +33 1 40 51 64 34; Fax: +33 1 40 51 64 30;

E-mail: jockers@cochin.inserm.fr

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Table 1 | Ligand-independent functions involving orphan seven-transmembrane proteins

G-protein-coupled receptor	Orphan 7TM protein	Effect on function	Figure 1	References
GABA _{B1}	GABA _{B2}	Export to the cell surface and G-protein coupling	(B)	White <i>et al</i> , 1998
DOR22a DOR43a	DOR83b	Export and improvement of functionality	(B) (C)	Neuhaus <i>et al</i> , 2005 Benton <i>et al</i> , 2006
T1R ₁ T1R ₂	T1R ₃	Effect on receptor functionality and receptor pharmacology	(E)	Nelson <i>et al</i> , 2001, 2002 Xu <i>et al</i> , 2004
MrgD	MrgE	Decrease of internalization Increase of ERK phosphorylation and intracellular [Ca ²⁺]	(B) (C)	Milasta <i>et al</i> , 2006
MT ₁	GPR50	Loss of ligand binding and function	(D)	Levoye <i>et al</i> , 2006
	ORF74	Constitutive activity responsible for oncogenic action	(F)	Rosenkilde <i>et al</i> , 2005
	UL33	Constitutive activity responsible for HCMV-related pathologies	(F)	Waldhoer <i>et al</i> , 2006
	EBI2	Constitutive activity in Epstein–Barr virus-infected cells	(F)	Rosenkilde <i>et al</i> , 2006
	Smo	Constitutive activity involved in embryonic development	(F)	Riobo <i>et al</i> , 2006

[Ca²⁺], calcium concentration; DOR 22a/43a/83b, *Drosophila* odorant receptors 22a/43a/83b; EBI2, Epstein–Barr virus-induced receptor 2; ERK, extracellular-signal-regulated kinase; GABA_{B1/B2}, metabotropic γ -aminobutyric acid (GABA) B receptor 1/2; GPR50, G-protein-coupled receptor 50; HCMV, human cytomegalovirus; MrgD or MrgE, Mas related gene D or E; MT₁, melatonin receptor; ORF74, (Open reading frame 74) Herpesvirus 8 encoded receptor; Smo, Smoothed; T1R_{1/2}, taste receptors 1/2; UL33, human cytomegalovirus chemokine receptor-like protein.

Further support for the role of orphan 7TM proteins in receptor transport to the cell surface comes from odorant GPCRs in insects, in which each olfactory sensory neuron (OSN) expresses a conventional ligand-binding odorant receptor together with *Drosophila* odorant receptor 83b (DOR83b)—a highly conserved odorant 7TM protein that has no apparent affinity for odorants. DOR83b has been shown to heterodimerize with classical odorant receptors early in the endomembrane system of the OSN. In addition, DOR83b is essential for the targeting and maintenance of odorant receptor heterodimers in sensory cilia membranes of OSNs and strongly increases the functional response of conventional odorant receptors (Benton *et al*, 2006; Neuhaus *et al*, 2005). These two examples illustrate the possible promoting effect of orphan 7TM proteins on the cell-surface expression of non-orphan GPCRs (Fig 1B; Table 1). The opposite effect—that is, intracellular retention of non-orphan GPCRs by orphans—has not yet been documented but is likely to exist because intracellular retention has already been identified for several non-orphan GPCR heterodimers (Prinster *et al*, 2005). Together, orphan 7TM proteins might indeed be involved in the regulation of GPCR cell-surface expression.

Modulation of receptor signalling and internalization

The ultimate function of GPCRs is to transduce extracellular signals across the plasma membrane. Two recent examples indicate that signalling through non-orphan GPCRs might be subject to negative and positive regulation by orphan 7TM proteins (Fig 1C,D).

The first example concerns the Mas-related gene (Mrg) family that comprises orphan and non-orphan members. The MrgD subtype, for instance, is activated by β -alanine, whereas the MrgE subtype is an orphan GPCR. Interestingly, both receptors are expressed in individual dorsal root ganglion neurons (Zhang *et al*, 2005). The detection of MrgD/MrgE heterodimers in these cells is limited by the lack of Mrg receptor subtype-specific antibodies, whereas heterodimer formation has clearly been shown in transfected human embryonic kidney (HEK)293 cells (Milasta *et al*, 2006). Coexpression in HEK293 cells increases the potency of the β -alanine-stimulated MrgD to phosphorylate extracellular-signal-regulated kinase 1 (ERK1) and ERK2 and enables β -alanine to maintain elevated concentrations of intracellular calcium (Fig 2A). Although the precise mechanisms leading to these effects remain elusive, these data highlight the importance of orphan

7TM proteins as positive regulators of non-orphan GPCR signalling. In addition, orphans might not only modulate the signalling of pathways that are activated by the homodimer, as shown for MrgD and MrgE, but also induce heterodimer-specific signalling events by recruiting additional intracellular regulators (Fig 1C).

MrgE regulates not only MrgD signalling, but also MrgD internalization (Milasta *et al*, 2006). When expressed individually in HEK293 cells, MrgD internalizes rapidly on β -alanine stimulation, whereas MrgE does not. However, expression of both receptors in these cells impairs ligand-promoted MrgD internalization (Fig 1D). Prolonged surface expression of MrgD in the presence of MrgE might contribute to the increased signalling potency of MrgD in the heterodimer.

We recently reported the existence of heterodimers between the orphan GPR50 and the MT₁ and MT₂ melatonin receptors in transfected HEK293 cells (Fig 2B), which constitutes another example of an orphan/non-orphan heterodimer (Levoye *et al*, 2006). MT₁ and MT₂ subtypes respond to the circadian neurohormone melatonin, whereas GPR50 has not been matched with any known ligand. However, structural evidence has placed GPR50 in the melatonin receptor subfamily (Reppert, 1997). Although GPR50 specifically interacts with MT₁ and MT₂, only the functional properties of MT₁ seem to be affected in the corresponding heterodimer. Indeed, the presence of GPR50 impairs signalling of MT₁ through the adenylyl cyclase pathway (Fig 1D). The formation of GPR50/MT₁ heterodimers completely inhibits high-affinity agonist binding as well as agonist-promoted G-protein coupling and β -arrestin-binding to the MT₁ protomer (Levoye *et al*, 2006). Interestingly, all these effects depend on the presence of the long (>300 amino-acid residues) carboxy-terminal tail of GPR50, which prevents the recruitment of intracellular interaction partners to MT₁ in the MT₁/GPR50 heterodimer. Coexpression of GPR50 and MT₁ transcripts can be observed in several cell types (A.L. & R.J., unpublished data), whereas the interaction of these two proteins has not yet been shown in tissues owing to the lack of appropriate tools. However, the potential significance of the inhibitory effect of GPR50 on MT₁ function is supported by the observation that endogenous GPR50 expression levels in human endothelial cerebral CMEC/D3 cells are sufficient to abolish MT₁ activity completely, whereas downregulation of GPR50 induces MT₁ signalling (Levoye *et al*, 2006).

Fig 1 | Possible ligand-independent functions of orphan seven-transmembrane proteins in heterodimers. **(A)** Signalling and internalization of G-protein-coupled receptor (GPCR) dimers with known ligands controlled by regulating molecules (including G proteins, β -arrestins, regulators of G-protein signalling, Homer family proteins and PDZ-domain-containing proteins). **(B)** Regulation of the export of GPCRs with known ligands to the cell surface by orphan seven-transmembrane (7TM) proteins. Heterodimerization might promote surface expression of the functional heterodimer or retain the heterodimer in intracellular compartments. **(C)** Increase or induction of the interaction of non-orphan GPCRs with intracellular regulators in the same or different signalling pathways (compared with the non-orphan GPCR homodimer) by orphan 7TM proteins. **(D)** Inhibition of receptor internalization or the interaction of non-orphan GPCRs with intracellular regulators by orphan 7TM proteins. **(E)** Modulation of the ligand-binding properties of the non-orphan protomer compared with the corresponding homodimer by orphan 7TM proteins. **(F)** Orphan GPCRs could have constitutive activity and thus ligand-independent functions. GPCRs with known ligand (pale pink); orphan GPCR (dark blue); intracellular regulators (turquoise); ligands (orange). ER, endoplasmic reticulum.

Modulation of receptor pharmacology

The description of more than 40 heterodimeric GPCR pairs (Prinster *et al*, 2005) has generated considerable interest among pharmacologists and biologists; not only are these new drug targets but also they could explain some basic biological processes, such as taste sensation. Taste receptors are class C GPCRs that are selectively expressed in taste buds and that mediate sweet and umami tastes. T1R₁ and T1R₂—originally discovered as orphan receptors—have subsequently been ‘deorphanized’ by the discovery of T1R₃, their obligatory heterodimerization partner. According to the current working model, taste receptor heterodimers are composed of either T1R₁ or T1R₂—which bind ligands with their extracellular Venus flytrap domain (VFT)—and of T1R₃, the corresponding VFT of which does not apparently bind to any ligand (Nelson *et al*, 2001, 2002; Xu *et al*, 2004). Binding of sweet stimuli such as aspartame to the VFT activates T1R₂/T1R₃ heterodimers, whereas the umami taste of L-glutamate activates the T1R₁/T1R₃ heterodimer in an analogous manner (Fig 1E). Furthermore, lactisole and cyclamate have been proposed to bind to an additional binding site located in the transmembrane domain of T1R₃ and thus allosterically regulate ligand binding to the VFT of T1R₁ and T1R₂ in the respective heterodimers. This indicates

that even though orphan 7TM proteins cannot themselves be regulated by ligands, they are possibly subject to allosteric regulation within a heterodimer. Similar observations have been made for GABA_{B2}, which is devoid of GABA-binding ability but which can be directly activated by the allosteric modulator CGP7930 (Binet *et al*, 2004). The presence of T1R₃ has marked effects on the ligand-binding properties of T1R₁ and T1R₂, but more subtle effects of orphan 7TM proteins are possible on the ligand-binding properties of the non-orphan GPCR.

Constitutively active orphan receptors

Several orphan GPCRs have been reported to be constitutively active (Fig 1F; Rosenkilde *et al*, 2006; Vischer *et al*, 2006). Consequently, these proteins have ligand-independent functions. Constitutive signalling is of major patho-physiological significance because various diseases are associated with GPCR mutations that lead to constitutive activation (Schoneberg *et al*, 2004). Interestingly, many of these constitutively active orphans are either virus-encoded or virus-induced, highlighting the capacity of viruses to use these receptors to reprogramme cellular functions after infection. For example, the constitutive activity of the human orphan herpesvirus-8-encoded receptor

ORF74 is responsible for its oncogenic potential to cause Kaposi's sarcoma-like lesions (Rosenkilde *et al.*, 2005; Sodhi *et al.*, 2004). Moreover, the human cytomegalovirus (HCMV) encodes three orphan GPCRs, of which UL33 is constitutively active in infected cells. Evolutionary conservation and promiscuous G-protein coupling of UL33 points to an important role for this receptor in HCMV-related pathologies (Vischer *et al.*, 2006; Waldhoer *et al.*, 2002).

Interestingly, the Epstein–Barr virus (EBV)-induced receptor 2 (EBI2), originally identified as the most upregulated gene in EBV-infected cells, is an orphan 7TM protein, which constitutively signals through the inhibitory (G_i) subunit of G proteins (Rosenkilde *et al.*, 2006).

Constitutive activity has also been shown for Smoothened (Smo), a G_i-coupled 7TM protein that mediates the effects of Hedgehog (Hh) in embryonic development. Normally, the constitutive activity of Smo is repressed by the 12TM receptor Patched (Ptch). Binding of Hh to Ptch relieves the repression of Smo. Consequently, the latter can be considered an orphan 7TM protein, the activity of which is regulated by its binding partner Ptch (Riobo *et al.*, 2006). The G_s-coupled receptor GPR3 that prevents premature ovarian ageing and maintains meiotic arrest in oocytes is another example of an orphan receptor with constitutive activity (Ledent *et al.*, 2005; Mehlmann *et al.*, 2004). However, it is important to keep in mind that the presence of unidentified endogenous ligands is always difficult to exclude for constitutively active proteins.

Finally, constitutively active orphan GPCRs could also be part of an orphan/non-orphan heterodimer and thus regulate the function of non-orphan GPCRs according to one of the above-mentioned regulatory mechanisms (Fig 1B–E).

Regulation of ligand-independent orphan 7TM proteins

Ligand stimulation constitutes a specific and highly controlled way to regulate the activation state of GPCRs. As orphan 7TM proteins, by definition, are devoid of this type of modulation, the control of the level of protein expression probably represents the main regulatory mechanism of these proteins. This could include regulation at the level of gene expression or by virus infection—through either the induction of target-cell genome-encoded receptors or the expression of virus-encoded receptors. Apart from the control of the absolute expression level, the relative expression of the orphan 7TM protein compared with the paired non-orphan receptor is also an important mode of regulation. This might be particularly true for orphans that inhibit the function of a non-orphan receptor, as increasing the quantity of the orphan 7TM protein would titrate the function of the non-orphan receptor. Finally, the orphan 7TM protein itself is, of course, subject to the well-known post-translational regulatory mechanisms involved in GPCR desensitization. Despite the absence of ligand binding, pharmacological intervention might still be possible by using allosteric ligands acting outside the classical ligand-binding site as detailed above.

How to identify orphan/non-orphan heterodimers?

The sequencing of the human genome has provided a comprehensive classification of all orphan receptors. Some of these receptors segregate into subfamilies exclusively composed of orphan receptors, but others (12 human and 49 mice orphan GPCRs) have been assigned to subfamilies with known ligands (Vassilatis *et al.*, 2003). Two further studies analysing sequences of the human rhodopsin GPCR subfamily or comparing human and *Drosophila* GPCRs came to a similar conclusion, placing more than 20 and 60 orphan

Fig 2 | Signalling properties of orphan/non-orphan heterodimers versus non-orphan homodimers. **(A)** Stimulation of MrgD homodimers with β -alanine promotes MrgD internalization, ERK activation and the transient elevation of intracellular calcium concentration. Expression with the orphan MrgE potentiates ERK activation, allows β -alanine to elicit a sustained increase in intracellular calcium levels, and inhibits MrgD internalization. **(B)** Stimulation of MT₁ homodimers with melatonin increases coupling to inhibitory G_i proteins and β -arrestin recruitment, and inhibits cAMP accumulation. Coexpression with the orphan GPR50 prevents β -arrestin and G_i protein interaction probably owing to steric hindrance caused by the long carboxy-terminal tail of GPR50. In the absence of functional G-protein coupling, the MT₁ protomer is devoid of high-affinity agonist binding. β -Ala, β -alanine; ERK, extracellular-signal-regulated kinase; GPCR, G-protein-coupled receptor; GPR50, G-protein-coupled receptor 50; MLT, melatonin; Mrg, Mas-related gene; MT, melatonin receptor.

receptors, respectively, within families that bind to known ligands (Fredriksson & Schioth, 2005; Metpally & Sowdhamini, 2005). Classifying orphan GPCRs into subfamilies with known ligands has been successfully used to deorphanize receptors in the past (Civelli, 2005). The fact that many of these classified receptors remain orphans supports the idea that some of these receptors might turn out to be 7TM proteins with ligand-independent functions.

In addition, it is interesting to note that for all known orphan/non-orphan heterodimers, the two protomers belong to the same receptor subfamily. Although this does not exclude the possibility of heterodimerization between distantly related 7TM proteins and non-orphan GPCRs, it clearly indicates that focusing the search on orphan receptors that have a high degree of sequence homology to non-orphan GPCRs is the most promising strategy for identifying physiologically relevant heterodimers.

Studying the coexpression profiles of orphan and non-orphan GPCRs could be another approach for identifying new orphan/non-orphan heterodimers. The brain is certainly the organ of choice for such a study as most GPCRs, including orphan GPCRs, are expressed in this tissue.

Concluding remarks and perspectives

The deorphanization of GPCRs continues to have a fundamental impact on our understanding of biological functions and on drug discovery. Although classical deorphanization processes have been successful, there is a need for alternative strategies and concepts to deorphanize the remaining orphan GPCRs. The identification of possible ligand-independent functions of orphan 7TM proteins through heterodimerization with non-orphan GPCRs might be one of these new methods.

This idea further increases the functional diversity of GPCR heterodimers as heterodimers might contain not only GPCRs with known ligands, but also orphan and/or constitutively active 7TM proteins. The further organization of these dimers in higher order structures—oligomeric arrays—might also be considered in this context. Future research should reveal the relevance of such a new concept by evaluating the number of orphan 7TM proteins that can be successfully paired with their dimerization partners. It is tempting to speculate that heterodimers composed of two protomers that respond to two different ligands—depending on the availability of one or the other ligand—could switch between ligand-dependent and ligand-independent functional modes. This could be an additional twist to the extraordinary diversity already offered by GPCR heterodimerization.

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