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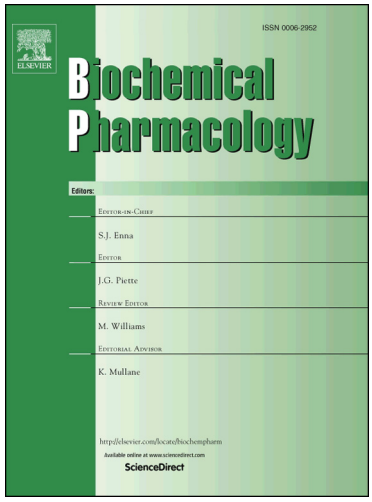

Review

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The G Protein-Coupled Receptors deorphanization landscape

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Abstract

G protein-coupled receptors (GPCRs) are usually highlighted as being both the largest family of membrane proteins and the most productive source of drug targets. However, most of the GPCRs are understudied and hence cannot be used immediately for innovative therapeutic strategies. Besides, there are still around 100 orphan receptors, with no described endogenous ligand and no clearly defined function. The race to discover new ligands for these elusive receptors seems to be less intense than before. Here, we present an update of the various strategies employed to assign a function to these receptors and to discover new ligands. We focus on the recent advances in the identification of endogenous ligands with a detailed description of newly deorphanized receptors. Replication being a key parameter in these endeavors, we also discuss the latest controversies about problematic ligand-receptor pairings. In this context, we propose several recommendations in order to strengthen the reporting of new ligand-receptor pairs.

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1. Introduction

Together with their cognate ligands, members of the seven transmembrane G protein-coupled receptors (GPCR) family are key component of numerous pivotal (patho)physiological processes such as neurotransmission, vision, cellular proliferation, development, pain, vascular homeostasis, muscle contraction or hormone secretion [1]. In addition, this group of receptors constitutes undoubtedly a productive source of drug targets [1]. The literature describing the targets for registered medicines proposes a proportion ranging from ~20 to more than 50% of drugs producing their therapeutic actions through the modulation of a GPCR [1-3]. The discrepancies in these numbers are probably a consequence of the varying definitions of the "target" (direct or indirect, individual receptors or families,...) or drugs (several non-overlapping databases for registered drugs exist). Recently, Sriram & Insel have tackled this issue by analyzing three public databases and carefully curated the receptors list. They concluded that 134 GPCRs were currently mediating the therapeutic effect of ~25% to ~33% of registered drugs [4]. Another recent extensive analysis by Hauser et al. suggested that ~34% of the FDA-approved drugs targeted 108 unique receptors [1]. These numbers place the GPCR family at the first place of protein families targeted by approved drugs. However, only a small portion (100-140) of the ~360 non olfactory GPCRs is currently exploited and the family is globally underused with regard to its potential in drug discovery.

Regarding GPCR-dependent propagation of the signal, a general paradigm has long been established. It proposes that these membrane receptors have the ability to adopt several conformations characterized by different affinities toward extracellular ligands and intracellular signaling partners [5]. In the presence of an activating ligand, a population with restricted conformations will be enriched by stabilization [6]. These restricted sets of conformations will

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promote the binding of receptor-specific G proteins. The various families of G proteins have the ability to activate distinct signaling pathways. For instance, Gs and Gi/o regulate intracellular levels of cyclic AMP through positive and negative modulation of adenylate cyclase, whereas Gq induces a rapid release of Ca^{2+} from intracellular stores after phospholipase C activation. The extinction of GPCR signaling is processed by desensitization, where the uncoupling of the receptor from its G protein occurs generally by GPCR kinase (GRK)-mediated phosphorylation of the intracellular C-terminal tail of the receptor [7]. Activation and phosporylation enhances the affinity of the receptor for the cytosolic adaptor protein arrestin that promotes receptor internalization through clathrin-coated pits [8]. It is noteworthy that arrestins have been proposed to initiate a cellular signaling independent of G protein activation [9,10]. However, this concept has been challenged recently with cells genetically depleted for arrestins or G proteins [11,12]. In addition, intracellular signaling from the endosomal compartment has been evidenced for GPCR, which seems to be driven by receptor-G protein-arrestin super-complexes [13,14].

From an historical perspective, ligands for GPCRs (adrenaline, serotonin, acetylcholine or morphine, to name a few) have been identified before their receptor counterparts, at a time when the concept of receptor itself was controversial [15]. Although many cognate receptors for endogenous ligands were rapidly cloned during the eighties and nineties, many investigators came rapidly across unidentified receptors [16] and labeled them as "orphan" for their endogenous ligands [17]. Since their conceptual definition, the number of orphan GPCR continued to grow significantly until the publication of the human genome sequence [18], the phylogenetic analysis of the human GPCR repertoire [19] and a detailed list of receptors issued by IUPHAR [20]. GPCR are usually classified by phylogeny and the most recent analysis proposed to group the receptors in families according to their resemblance to Glutamate,

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Rhodopsin, Adhesion, Frizzled or Secretin receptors, termed the GRAFS system [19]. A former classification is often found in the literature and assigns the different receptors into classes that roughly overlap the GRAFS categories: class A (Rhodopsin-like), class B (Secretin-like) and class C (Glutamate-like). Not all the families contain orphans, most of them are present in the Adhesion, Rhodopsin (Class A) and Glutamate (Class C) families. A list of orphans is maintained on the receptor database established by a shared effort from the International Union of Basic and Clinical Pharmacology (IUPHAR) and the British Pharmacological Society (BPS) [21]. For example the numerically largest Rhodopsin-class A family totalizes 91 orphan receptors.

According to the recommendations of the IUPHAR [22], a receptor is considered "deorphanized" when i) two or more peer-reviewed papers from independent labs report activity of the ligand at the receptor, at potencies compatible with a physiologic function. The assays describing pharmacological activity should be diverse and measure different parameters (affinity with binding assays and second messengers for determination of efficacy and potency). The reproducibility criterion is of prime importance and discrepancies should be carefully analyzed and explained. It can become problematic when two independent labs report a ligand for a receptor but other fail to confirm the result. When such controversies arise, the IUPHAR encourages further investigations before making a definitive statement. ii) The proposed endogenous ligand must be present in the tissues at sufficient levels. Several analytical techniques such as mass spectrometry or radioimmunoassays can be used to measure the concentration of small molecule ligands in tissues. The use of genetically engineered mice lacking the receptor should display a phenotype that is in accordance with the proposed pharmacological link between an endogenous ligand and its cognate receptor.

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When robustly demonstrated, the identification of an endogenous ligand for an orphan receptor is often the key to understand a novel physiological process. In addition, such discovery opens new avenues in terms of drug discovery. One of the most recent success stories from initial discovery to drug development is the identification of the orexin system. Orexins are 28- and 33-amino acids long peptides located in the hypothalamus and were identified as ligands for the orphans HFGAN72 and D81887 (OX1 and OX2 receptors, respectively) in 1998 [23]. The newly discovered peptides and their receptors were named in reference to the greek word for "appetite" (orexis). However, subsequent investigations demonstrated their importance in the physiology of sleep [24]. In 2014, an orexin receptor antagonist, suvorexant, has been approved by the FDA for the treatment of insomnia (Belsomra®, Merck), a condition with important unmet medical needs [25]. At that time, the only efficient drugs were $GABA_A$ positive modulators such as benzodiazepines and related molecules (such as Zolpidem). Therefore, suvorexant, as a dual-orexin receptors antagonist, laid the foundation of a novel class of medicine. Moreover, it hit the market less than 20 years after the orexin receptors have been deorphanized, thus demonstrating the importance of this kind of research in terms of drug discovery.

In this review, we will present the traditional and actual strategies to study orphan receptors and identify their ligands. An extensive description of the orphan GPCR field has been published in 2013 by Davenport *et al.* [22]. Therefore, we will focus on the deorphanizations that were reported since 2013. Because of the importance of the reproducibility of initial pairings, we also discuss prominent ongoing controversies regarding problematic orphan GPCR pairings.

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2. Current strategies to assign function to orphan GPCR

Each orphan GPCR bares the potential of being the kick-start of a whole new research area with novel therapeutic options. However, the identification of a clearly defined function for the remaining orphans is a daunting task and these understudied receptors must be extensively investigated with innovative tools in preclinical research before reaching the status of validated target. There are many hurdles that preclude research on orphan GPCRs and the field is obviously suffering from a "streetlight effect" , where investigators are biased toward the same well-described receptors, just because there are tools to study them. This phenomenon is not restricted to GPCR and is observed in many other fields [26]. Therefore, the relatively limited literature on some orphans is probably the consequence of a paucity of useful probes and adequate tools [27]. An additional possible explanation for lack of literature expansion on particular orphans is that attempts by other labs to reproduce the receptor-ligand pair failed, thus precluding further research initiatives on the topic. For investigators interested by obscure orphans, the very first step is to carefully design and generate innovative tools to address the questions about the receptor function. Schematically, three types of experimental strategies can be envisaged: a pharmacological approach, a genetic approach or both combined. Strangely, whereas the identification of a ligand is usually followed by studies in transgenic models and the characterization of a phenotype followed by the quest for a ligand, strategies build on both approaches simultaneously are not so common, although they would be probably highly efficient. We present in this section the current trends in technologies applied to orphans and discuss their respective advantages and weaknesses.

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2.1. Pharmacological approaches

The gold standard: the endogenous ligand

The traditional fast track to assign a function to an orphan receptor and to validate its potential as a drug target is to identify its endogenous ligand(s). However, the quest for these elusive entities can be challenging and there are probably numerous unreported failures of such endeavors. In 2008, Levoye & Jockers noticed that the rate of published deorphanizations was slowly decreasing [28]. We updated this graph and we observed that this trend has been confirmed over the past 10 years (Fig. 1). However, although at an historical low, the rate is not decreasing anymore and novel ligand-receptor pairs are identified at constant pace, with some years better than other. Most notably, 2016 has seen a relatively high number of advances in the field (see below). Therefore, rather than a crisis, we may argue that we are actually beyond the "orphan GPCRs hype" and that the field has entered the age of maturity. In other words, the low-hanging fruits may have all been collected and there is now a need to develop new approaches to continue to make significant discoveries. Therefore, we can hypothesize that the deorphanization process will now continue at a constant, although reduced, pace (Fig. 1).

Regarding the remaining orphans, several reasons can be put forward to explain the current absence of known natural ligands [22,28]. First of all, it is not clear whether an endogenous "ligand" actually exists for all orphan GPCRs. In fact, some may exert their function in a ligand-independent fashion by being constitutively active or by modulating other GPCRs (or other proteins) [22,29]. One well-described mechanism of a ligand-independent function is the modulation of a receptor through heterodimerization. For example, GPR50 has been shown to prevent melatonin receptor MT_1-mediated agonist binding and G protein coupling through heterodimerization [30]. Another example is the Mas-related receptor MrgE that potentiates the

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signaling of its interacting partner MrgD when stimulated with its ligand β-alanine [31]. Even if the hypothesis that receptors could have ligand-independent function is true, it can't be experimentally proven that a receptor has no endogenous ligands because absence of evidence of the existence of a ligand is not evidence of absence of such ligand.

If the receptor has a ligand, it should be present at some point in the organs where the receptor is expressed. This obvious fact was the conceptual justification of assaying the orphan receptors with tissue extracts, which is the traditional source for endogenous ligands. A potential issue of this approach is that the isolation of the endogenous ligands from tissue extracts can be challenging if they are tightly regulated and/or transiently produced, at very low level or highly unstable. Therefore, if the endogenous ligand is an unknown transmitter it is an additional challenge to isolate and characterize it.

Another possible source of failure in deorphanization campaigns is the choice of the assay to detect receptor activation. If the ligand is present in the tissue extract, current paradigm postulates that the GPCR will respond if placed in a generic "GPCR assay". These "universal" assays are based on general signaling mechanisms observed in most of the GPCRs, such as arrestin recruitment or G protein coupling. The assay is designed based on the assumption that the orphan receptor will behave just like other closely related receptors. This traditional strategy was recently used to implement a deorphanization platform based on an arrestin recruitment assay [32]. However, some receptor-ligand pairs may employ currently unknown or alternative signaling mechanisms different from the canonical GPCR pathways and will therefore be “missed” in traditional GPCR screening methods (for an extensive description of peculiar GPCR pathways, see Ritter & Hall [33]). Similarly, screening campaigns of tissue extracts may have failed due to an incomplete receptor system in cell-based assays such as absent interacting

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partners if the receptor needs complimentary proteins to be fully functional. For example, the calcitonin receptor (CTR) and the CT Receptor-like (CLR) acquire new signaling and ligand binding properties in the presence of Receptor Activity-Modifying Proteins (RAMP) [34]. The pharmacological profile of the endogenous ligand can also be problematic. Although we usually think of a ligand as an agonist, it may well be for instance an inverse agonist (only one reported example exists with the Agouti-Related protein and MC4R [35]). If the assay is not designed to detect this kind of behavior, the ligand may be missed. In fact, the hunt for a ligand should ideally start with the identification of the receptor signaling pathways. Then, an optimal assay should be developed or implemented in order to gain confidence in the fact that once the agonist ligand and the receptor will be mixed together, a signal will be recorded. The importance of the assay choice is illustrated by cases of receptors with atypical coupling, such as a presumed absence of G protein coupling (CXCR7 [36], C5aR [37] or LGR5 [38]), arrestin recruitment (Beta 3 adrenoceptor, B3AR [39] or the glucose-dependent insulinotropic polypeptide, GIP receptor [40]) or both (such as the Angiotensin Receptor type 2, AT2R [41]). In a recent example we could not find evidence for G protein coupling of the orphan GPR27, an observation that may explain the previous failure of deorphanization campaigns for this receptor [42,43].

The complete description of the full array of GPCR assays that can be implemented is out of scope for the present review but interested readers will find information in comprehensive review focused on assays [44].

Bypassing the endogenous ligand: Surrogate ligands

Because of the relative decrease of successful deorphanization campaigns, the option to bypass the endogenous ligands has logically gained popularity over the past few years. Interestingly, the available techniques to characterize a protein function have reached such a level

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of sophistication that some GPCR have gained the status of drug target and have seen programs of drug development started while they were still orphans. For instance, modulators for GPR119, GPR35, GPR55, MAS or GPR84 have entered clinical trials for various conditions [1].

Thus, even if the endogenous ligand is unknown (if there is one), crucial information on the pharmacology of orphan receptors can still be obtained by using "surrogate" ligands, or even other techniques (see below). Non-endogenous ligands (peptides, small molecules, antibodies etc.) can be used as probes to investigate a receptor function and/or its therapeutic potential [45]. They can be employed to identify signaling pathways and activate the receptor in an in vivo context. An additional advantage of this strategy is that even if the receptor has no endogenous ligand, its activity can -theoretically- still be modulated by small molecules or other ligands. However, surrogate ligands are no substitutes of the endogenous one, mainly due to the fact that they may favor different conformations and display different signaling profiles, trigger other effects or bind an allosteric site [46]. However, they have proven their effectiveness for discovering orphan receptor functions [47]. Therefore, they should be seen as a complementary, not alternative, approach in the quest for endogenous ligands, which remains the ultimate goal. There are actually several examples in the literature where surrogate ligand paved the way to the identification of the endogenous ligand-receptor system, by the identification of pharmacophores or by giving access to competition assays (such as binding with labeled ligands). For instance, synthetic ligands for the ghrelin receptor existed already back in the seventies, well before the receptor was known [48]. These ligands, called Growth Hormone secretagogues due to their endocrine effect, permitted the identification of the receptor and the endogenous ligand, ghrelin [48].

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The strategies implemented to identify surrogate ligand are essentially similar to those used for endogenous ligand (an adequate assay, generic or specific), the only difference being that the source of ligands suffers from less limitations. Many diverse libraries are now commercially available for screening. In addition, promising strategies based on tridimensional structures, homology modeling and virtual screening have been made available to the ligand hunter toolbox. These developments are the logical consequence of the recent explosion of crystal and cryo-EM structures for receptors (for readers interested by technical aspects, many good reviews have been published on this topic [49]). Actually, it seems that this particular field has reached momentum and is starting to deliver its promises. For example, Ngo *et al.* have recently managed to identify ligands for GPR37 by applying an elegant "pickpocketing" strategy consisting in comparing receptors with respect to their modeled ligand binding pockets instead of whole sequence homology [50]. Other investigators managed to identify surrogate ligands for the proton sensing receptors GPR68 and GPR65 by combining wet and virtual screenings [51]. These approaches complement previous attempts to classify receptor on other elements than pure sequence homology such as their pharmacology [52]. These strategies based on virtual screening are quite attractive because failure is relatively cheap compared to the resources needed to screen actual ligands. All the structural and computer-based strategies have been extensively reviewed elsewhere [47,53,54].

Surrogate ligands have another characteristic worth to mention: the possibility of functional selectivity (also termed biased agonism) [55] as this ligand-receptor property can play important function in the deorphanization process. Functional selectivity refers to the fact that different molecules can stabilize distinct conformations of receptors and hence promote one pathway over another, by modulating the affinity of the ligand-receptor complex toward

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downstream partners [55]. This very important aspect should be taken into account when selecting the screening assay as some may favor compounds that activate only one given pathways [56]. In addition, this property should be carefully addressed for each surrogate ligand in order to validate the conclusions that can be established when using such tool. If the compound is biased, its use won't reflect what would happen with a receptor activated by an endogenous ligand, unbiased by definition (as the endogenous is used as the reference to determine bias) [57].

It is noteworthy to mention that, although surrogate ligands are usually small molecules, the use of antibodies as pharmacological tools and therapeutic agents for GPCRs has attracted much attention over the past few years [58]. Nowadays, many marketed drugs are "biological" and these new kind of ligands have been used extensively in crystallographic studies on GPCRs. Theoretically they can also be used to stabilize different receptor conformations and act as (inverse) agonists or antagonists [59,60]. Although there are currently no reports describing such tools for orphan, the technology is susceptible to play a significant role in the near future. Other tools such as pepducins have been described as useful probes to characterize the function of orphan GPCR [61].

2.2. Functional genetics-based approaches

Progresses in molecular biology techniques have made possible to gain valid information about receptor signaling and function without the need of a ligand, endogenous or surrogate. The identification of function can be achieved by generating gain- (by modulation of constitutive activity of the receptor) or loss-of-function mutants and subsequent analysis of phenotypes. Alternatively, the signaling pathways can be inferred from protein interaction studies, performed by co-immunoprecipitation or BRET/FRET studies. However, these techniques are in general only useful for expected interactions, not for screenings. For this latter purpose, yeast-2-hybrid

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screens were shown to be a successful approach [62], but have the disadvantage of using highly artificial cellular environment. Alternative proteomic approaches – coupled to mass spectrometry - for detecting GPCR protein complexes are typically based on the use of either the entire GPCR or a part of it as bait. In the first case, the double tagged entire GPCR can be purified with its interacting proteins by tandem-affinity purification under mild conditions from a natural cellular environment [63-65]. In the second case, a chemically synthesized tagged subdomain of the GPCR is used as bait to bind interacting proteins from cell or tissue lysates, before purification by peptide affinity chromatography [66,67].

Phenotypical characterization of animals in which an orphan receptor is silenced or overexpressed can provide evidence for its physiological or pathophysiological function. Actually, KO mice are known to exist for more than one third of the remaining non-odorant orphans, of which only slightly more than twenty have a reported phenotype [22]. Importantly, the absence of an obvious phenotype at first sight should not be interpreted as a minor role of the receptor, as sometimes mice can seem normal unless they are exposed to a very specific condition where the ligand is produced. A somehow famous example of this is represented by β2AR KO mice, that appeared grossly normal, fertile and without physiological perturbations, until exposed to exercise stress [68]. Needless to say, finding this specific condition can be as challenging as finding a ligand for the receptor. This complexity is likely to be one of the reasons why no phenotype has been reported for an important number of receptors. Mild phenotypes are another limitation of this approach as redundancy in GPCR may compensate for the most critical function. A complete description of the non-pharmacological approaches-based research is beyond the scope of this review. However, a complete repertoire of current ligand-independent research has recently been established [46].

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The constitutive activity of a GPCR can be defined as its propensity to activate a given signaling pathway independently of the presence of a ligand. This peculiar phenomenon has been evidenced with the evolution of pharmacological bioassays that have seen their sensitivity reach exquisite levels. The existence of constitutive activity was highlighted short before the identification of inverse agonists as the two phenomena are conceptually linked. In the frame of the conformational ensemble, the constitutive activity is the consequence of a more important proportion of active conformations and an inverse agonist has the ability to stabilize inactive states of the receptor [69]. In the field of orphan receptor the constitutive activity can be of great help to decipher the function of the orphan. It can be used to spot the intracellular proteins (most usually G proteins and arrestins) to which the receptor is coupled in heterologous systems. The knowledge of the expected pharmacology for a given receptor can be the basis for the development of screening assays that have an increased sensitivity. This may facilitate the identification of a ligand. In the absence of such properties, mutants (Constitutively Active Mutant or CAM) may be generated to modulate the activity of a given receptor [28,46,70].

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3. Current deorphanization landscape

In this section, we present a detailed overview of the current state of ongoing investigations on recently deorphanized receptors. We also discuss some of the ligand-receptor pairs that have not yet reached consensus due to divergent data among different labs (see also Table 2).

3.1. Recent deorphanizations

Although the deorphanization pace has decreased (see above and Fig. 1), there are regular reports of endogenous ligand identification. We will limit the discussion on the claims of orphan receptor-ligand pairing that have appeared in the literature since 2013 (see Table 1 and Figure 1). For previously reported deorphanizations, interested readers may look at some of the comprehensive reviews that have been written on the topic prior to 2013 [22,71].

GPR15 - GPR15L

The interest for GPR15 as a putative drug target can be traced back to its potential role as viral co-receptor for the HIV and simian immunodeficiency virus [72]. Moreover, GPR15 expression is upregulated in some HIV-1 infected individuals and in HIV-1 infected $CD4^+$ T cells thus conferring susceptibility to the virus infection [73,74]. In 2013, several teams independently identified a function for this receptor in the T cell homing in the skin and intestine [75,76]. Since these seminal discoveries, other evidences of its involvement in T cell homing have been described in the colon [77,78]. Recently, a putative natural ligand (GPR15L) has been identified and shown to be able to modulate lymphocyte recruitment to epithelia in a GPR15-dependent fashion [79]. GPR15L, a 9KDa chemokine is encoded by the C10orf99 gene in humans and is expressed in organs where GPR15 is suspected to have a role in T cell homing such as mucosal

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surface and skin [79,80]. Collectively, these data on GPR15 and its putative ligand strongly suggest that it could be an attractive drug target in immune homeostasis disorders as well as mucosal and cutaneous inflammation.

GPR35 - CXCL17

Genetic association studies and GPR35 localization have suggested a role for GPR35 in inflammation, asthma, hypertension, and diabetes [81]. GPR35 is expressed at a high level in human pancreas and small intestine, but also in immune cells such as dendritic cells and monocytes [82,83]. Two variants of GPR35 are expressed in humans, a short and a long version with 31 additional amino acids at the N-terminal part of the receptor [84]. The physiological impact of the N-terminal extension is not solved, as similar in vitro effects have been observed for both GPR35 variants [85]. Historically, GPR35 has been claimed to be a receptor for kynurenic acid, an intermediate metabolite of the amino acid tryptophan localized in the brain and several peripheral tissues such as intestine, pancreas, lung, spleen, kidney and muscle [81,82]. It was soon noticed that the potency of kynurenic acid showed important discrepancies between species, being more active on the rat receptor compared to the human version [82]. In addition, the pharmacological responses of kynurenic acid are in the millimolar range for the human receptor in terms of EC_{50}, whereas it is present at micromolar concentrations in blood [86,87]. Therefore, the relevance of kynurenic acid as an endogenous ligand has long been questioned [81]. In line with the possibility of other more relevant ligands, CXCL17 was identified recently as an agonist for this receptor, opening a new range of hypothesis on the function of GPR35 [88]. Although this recent study pointed to structural similarities of GPR35 with chemokine receptors, other reports placed it in a distinct cluster [50]. The CXCL17-GPR35 axis has also been studied in breast cancer and a higher proliferation of breast cancer cells has

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been correlated to GPR35 expression together with CXCL17-induced proliferation and migration of cancer cells [89]. Another study by Park et al. questioned the activation of GPR35 by CXCL17. They used various methods, including cells expressing GPR35 endogenously (THP-1, a human monocytic leukemia cell line). The authors showed that GPR35 acts as an inhibitor of cell migration while CXCL17 promotes THP-1 cells migration in a GPR35-independent manner. In addition, they did not observe GPR35 activation with CXCL17 although they confirmed activation with other GPR35 agonists such as lodoxamide, pamoic acid, kynurenic acid and zaprinast in an AP-TGFα shedding assay [90]. Therefore, GPR35 has an important potential as a drug target but its pharmacology and the precise nature of its physiological ligand(s) remain elusive [83].

GPR139 - L-tryptophan and L-phenylalanine

This rhodopsin-like/Class A orphan is a representative example where information on surrogate ligands and binding pocket similarities were used to drive the discovery of the endogenous ligands. First, two groups independently identified different families of synthetic agonists for GPR139 [91,92]. In an elegant approach, Isberg *et al.* developed a pharmacophore model based on the structure of known ligands [93]. The following computer-based virtual screening around the scaffold identified aromatic amino acids dipeptides as potential ligands for the receptor [93]. The authors reasoned that individual amino acids could contribute to the activity of dipeptides and evaluated all L and D-amino acids in their assays. They subsequently showed that L-tryptophan and L-phenylalanine activated the receptor and proposed them as the probable endogenous ligands [93]. Several teams have confirmed L-tryptophan and L-phenylalanine as activating ligands for GPR139 and new screening campaigns have been initiated in order to improve pharmacophore modeling [94-96]. More recently, Nohr *et al.* performed a

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mutagenesis study that revealed important amino acids and proposed a binding site for L-tryptophan and L-phenylalanine [97]. In another recent study, the same group spotted similarities between the binding pocket of GPR139 and the melanocortin 4 receptor (MC4R) [98]. Accordingly, they demonstrated that GPR139 could be activated by endogenous MC4R agonist peptides adrenocorticotropic hormone (ACTH), α-melanocyte stimulating hormone (α-MSH), β-melanocyte stimulating hormone (β-MSH) and their conserved motif HFRW [98]. Interestingly, predicted cleavage product (α-MSH1-9) of the pre-pro-protein pro-opiomelanocortin (POMC) was also able to activate the receptor, in the submicromolar range. The physiological relevance of these observations is not clear at the moment. However, GPR139 being strongly expressed in the hypothalamus and striatum [94], it is tempting to speculate that it may have a role in metabolism and/or locomotor activity and could represents a novel drug target for metabolic disorders or Parkinson's disease. Accordingly, with the emergence of putative ligands for this receptor, the involvement of GPR139 in locomotion begins to be studied using agonist and antagonist in an in vitro Parkinson's model with primary culture of dopaminergic neuronal degeneration. In a particular study, GPR139 was found to have neuroprotective effects toward neuron degeneration [99].

GPR101 & GPR173 – $GnRH_{1-5}$

The decapeptide Gonadotropin releasing hormone, or GnRH, is a hypothalamic neuropeptide released in a pulsatile manner to control the production of LH and FSH from the anterior pituitary [100]. GnRH(1-5) is a trunctated, metabolized version of this peptide that seems to possess biological activities distinct from those of the full length GnRH [100]. In 2013, GPR173 was proposed to be the cognate GPCR mediating the effect of GnRH(1-5) on cellular migration of immortalized GnRH neurons (GN11 cells) via STAT3 [101]. GPR173 is a

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Rhodopsin-like receptor which is a member of a small cluster of 3 orphans, the super conserved receptors expressed in the brain (SREB) [43]. GPR173 has currently no assigned function. Although there were some indications that the GN11 GnRH neurons migration is G protein-mediated, no effects on cAMP or IP_3 levels could be observed. In contrast, β-arrestin 2 was robustly recruited to GPR173 in these cells [102]. Therefore, the authors proposed a mechanism different from the canonical G protein pathways to explain the effect of GnRH(1-5) on cellular migration [102]. In follow up studies, the same team proposed that GPR101, a non SREB member of the Rhodopsin-like receptor family, was mediating transactivation of EGF receptor by GnRH(1-5) in Ishikawa Human Endometrial Cells [103]. Although no precise function for GPR101 is known currently, it was recently shown to be involved in endocrine diseases such as gigantism and acromegaly [104]. The pairings have been reported in publications from a single team and independent investigators should confirm the relevance of these observations.

GPR83 - PEN and GPR171 – big LEN

Many neuropeptides have been characterized but for some of them, the receptors mediating their effect remain enigmatic. One team identified recently two related neuropeptides as endogenous ligands for two orphan receptors, GPR83 and GPR171. PEN has been identified as putative endogenous ligand for GPR83 and big LEN for GPR171 [105,106]. These two neuropeptides are produced from the same precursor and are co-secreted. PEN and big LEN have been described as having a function in feeding [107]. Therefore Gomes et al. tested the capacity of these two neuropeptides to bind to orphan GPCR selected as good candidates for having a function in metabolism regulation [105,106]. Indeed, GPR83 and GPR171 are expressed in brain areas involved in energy metabolism control such as hypothalamic nuclei [105,108]. In addition, GPR83 expression in the hypothalamus is modified when mice become obese [109]. GPR83

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forms heterodimers with the ghrelin receptor (Ghsr1a), which leads to a decrease in the activation of the receptor when stimulated with acyl-ghrelin [109]. Accordingly, GPR83 KO mice display a potentiated orexigenic effect of ghrelin [109]. Interestingly, in addition to the modulation of food intake through Ghrs1a, GPR83 KO mice are resistant to High Fat Diet (HFD)-induced obesity suggesting that GPR83 influences the systemic metabolism through ghrelin independent action [109]. Moreover, GPR83 forms functional hetero-oligomers with other GPCR, including GPR171 [110]. These two receptors are expressed in the same part of the brain and colocalize in some regions [106]. Accordingly, coexpression of GRP83 and GPR171 in cell lines produces alteration of signaling pathways activated by the receptors and cAMP inhibition mediated by the activation of GPR171 by big LEN is increased in GPR83 deficient mice [106]. Furthermore, a selective GPR171 synthetic agonist has been discovered and allowed the validation of its role in food intake and body weight [111]. Beside food-related effects, GPR171 has been studied in anxiety and fear models because GPR171 is expressed in the basolateral amygdala [112]. Very recently, an antagonist able to block GPR171 activation by big LEN has been identified following a virtual screening approach [112]. In this study, both GPR171 antagonist injection and GPR171 knockdown in the basolateral amygdala reduced anxiety behavior and fear conditioning [112]. A potential function of GPR171 in lung cancer cell proliferation and metastasis has also been pointed out recently [113]. The existence of several pharmacological tools for this receptor should facilitate the confirmation of this effect.

GPR37 & GPR37L1

GPR37 and GPR37L1 are two closely related GPCR expressed in the central nervous system. GPR37 is mainly expressed in neurons whereas GPR37L1 is exclusively expressed in glial cells [114]. They were clustered in the same family of orphan GPCR because they are at

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48% identical to each other [115]. GPR37 is the substrate for the E3 ubiquitin ligase parkin and mutations of this ubiquitin ligase lead to neuronal degeneration and Parkinson's disease [116]. In addition, GPR37 (also called Pael-R) forms aggregates in the cytoplasm when it is overexpressed and its presence in Lewy bodies suggests that it could be involved in neuronal cell death and Parkinson's disease [117].

Methods based on phylogenetic comparisons have first connected GPR37 and GPR37L1 to endothelin receptors [118]. However, neither endothelin nor related peptides activate GPR37 or GPR37L1 [115]. A new approach to classify GPCRs, called GPCR-CoINPocket, compared the binding pocket sequence of well-characterized GPCR crystallographic structures with orphan GPCR to try to identify surrogate or cognate ligands [50]. This system predicted that GPR37L1 was closer to orexin, bombesin and neuropeptide S receptors than endothelin receptor when comparing the binding cavity instead of full sequence. This similarity was confirmed experimentally with the discovery that different antagonists of orexin and neuropeptide S receptors acted as inverse agonists on GPR37L1 [50]. It has also been suggested that the high level of GPR37L1 constitutive activity was due to the presence of a tethered ligand located in the N-terminal part of the receptor [119].

Two cognate endogenous ligands have been proposed for GPR37 and GPR37L1 but so far none of them were unambiguously confirmed. The neuropeptide head activator (HA) was the first putative ligand described for GPR37 [120]. Originally, HA was isolated from hydra but HA identical sequence was also isolated from mammalian brain [121]. However, the gene coding for HA was not identified in human. Rezgaoui et al. observed an additional effect of HA on CHO-K1 cells stably expressing GRP37 in a Ca^{2+} mobilization assay using an aequorin biosensor [120]. However, a dose response curve was also detected in cells nontransfected with GPR37 [120].

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Furthermore, GPR37 internalization and Ca^{2+} release induced by HA were supported by Gandia et al. using microscopy and a NFAT-Luc reporter [122]. Other teams investigated the activation of GPR37 by HA, but didn't succeed to detect GPR37 internalization, ERK phosphorylation or cAMP modification [123,124]. Prosaposin and prosaptide are the two other putative ligands for GPR37 and GPR37L1. Prosaposin and prosaptide promoted GPR37 internalization, $ERK_{1/2}$ phosphorylation and inhibited cAMP production in a PTX-sensitive manner [125]. Neuroprotective effects of prosaptide were reduced when GPR37 was knockdown with siRNA [125]. However, different investigators were unable to recapitulate the results obtained with prosaposin and prosaptide on GPR37 [50,124]. Recently, GPR37L1 has been suggested to mediate the role of prosaptide in the inhibition of astrocyte glutamate uptake [114]. Although no direct evidence of binding of prosaptide to GPR37L1 was provided, this effect seemed to be specific to GPR37L1 as no signal was observed for astrocyte derived from GPR37L1 KO mice [114].

GPR64, GPR133, GPR126 and GPR114

A particular type of ligand that has attracted attention these last years is the tethered agonist. Classically, it is an amino acid sequence located in the N-term of the GPCR that has the ability to activate the receptor. The presence of the agonist in the sequence of the receptor was well described already for protease activated receptors (PARs) such as the receptor for thrombin [126]. The protease cleaves a N-terminal ectodomain that reveals a tethered agonist sequence that can bind and activate the receptor [127]. More recently, GPR37L1 was shown to function in a similar fashion [119]. In the past few years, tethered agonists have been described for four orphan GPCRs belonging to the family of adhesion GPCR (aGPCR): GPR64, GPR114, GPR126 and GPR133 . The mechanism for aGPCR activation is similar to PAR receptor except that, at least

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for these 4 receptors, aGPCR expose their ligand sequence without the involvement of a separate protease. Actually, all aGPCR except one (GPR123) display a complex N-terminus containing a GPCR-autoproteolysis inducing domain (GAIN) cleaving the ectodomain at a highly conserved GPCR proteolytic site (GPS) [128]. aGPCR are usually involved in cell-cell or cell-matrix interactions but beside their adhesion function, this type of receptor is also able to transduce a signal by a G protein dependent or independent pathway [129]. Tethered agonists for GPR64, GPR114, GPR126 and GPR133 have been studied most notably using targeted mutagenesis approaches. In 2014, Liebscher et al. identified a N-terminus deleted form of GPR126 and GPR133 having increased Gs activity compared to wildtype receptor [130]. The deleted N-terminal part is composed of the sequence upstream of the GPS allowing the exposition of a sequence named "Stachel" located in the GPS and specific to the receptor activation. Indeed, amino acids sequences derived from this Stachel sequence have been directly tested on the receptor deleted for the Stachel sequence and a restoration of the Gs coupling was observed for this mutant. The mutation of two amino acids in the Stachel sequence of GPR126 disturbs myelination of peripheral axons in zebrafish, a result in line with data obtained in mice depleted for GPR126 [130,131]. A functional interaction with laminin has also been suggested for GPR126 [132]. Using similar mutagenesis strategies, a Stachel sequence has been also identified for GPR64 [133] and GPR114 [134]. GPR64 has a role in male reproductive system and is proposed as drug target to treat infertility whereas no function has been assigned to GPR114 yet [133].

It is not clear whether this mechanism of activation is common to all aGPCR. Current hypothesis on activation postulates that the exposure of these intramolecular agonists is the consequence of structural rearrangement induced by the binding to an extracellular ligand

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through the N-terminal part of the receptor [130]. Regarding these Stachel sequences, it is interesting to note that the one that activates GPR126 cannot activate GPR133 and vice versa [130]. However, it has been recently described that peptides derived from GPR110 Stachel sequence can also interact with GPR64 and GPR126 giving a certain level of promiscuity of some Stachel-derived peptides [135].

In addition to having a tethered ligand, GPR126 was also proposed to be the receptor for the cellular prion protein PrP(C) [136]. An increase of cAMP was observed in primary Schwann cells expressing GPR126 in presence of PrP(C). The Schwann cell line SW10 deleted for GPR126 did not lead to similar cAMP level increase [136]. Additional studies by independent labs should be performed to confirm these observations.

GPR75 - 20-HETE

In 2006, Ignatov et al. identified the inflammatory chemokine RANTES/CCL5 as activator of the orphan GPR75 [137]. They used as a read out the induction of Ca^{2+} mobilization and the generation of inositol triphosphate (IP_3) [137]. In addition, they observed that the endogenously expressed GPR75 in HT22 hippocampal cell lines has neuro-protective effect against cell death induced by β-amyloid peptides exposure [137]. However, since this seminal finding, the pairing of RANTES and GPR75 has been substantiated only once by a study suggesting a role of GPR75 in insulin secretion in human and mouse isolated islets [138]. Moreover, a screening of natural ligands on orphan GPCR with a β-arrestin recruitment assay failed to confirm the activation of GPR75 by RANTES [124]. Recently, 20-Hydroxyeicosatetraenoic acid (20-HETE) has been proposed as a novel ligand for GPR75. It is

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the first cytochrome P450-derived eicosanoid of its class to be referred as a ligand for a GPCR [139]. The elevation of 20-HETE in smooth muscle and endothelial cells has been associated with risk of hypertension, myocardial infarction and vascular diseases [140]. Garcia et al. used click chemistry and crosslinking analogs to identify a receptor that could explain 20-HETE effects on vascular function and blood pressure [139]. The putative 20-HETE-GPR75 interaction was shown to activate the Gq/11 pathway and led notably to elevation of IP_3. The effect on the vasculature was suggested to be the consequence of c-Src-mediated transactivation of epidermal growth factor receptor and induction of angiotensin-converting enzyme expression. Garcia et al. also demonstrated in a transgenic mouse model that GPR75 was required to observe 20-HETE-induced hypertensive effect and angiotensin-converting enzyme induction [139].

GPR107 – neuronostatin and GPR146 - proinsulin

In a couple of recent investigations, Yosten et al. applied a similar strategy for the identification of orphan GPCR that could pair with neuronostatin and proinsulin C-peptide [141]. These two peptides had a well-described physiological role but the receptor mediating their functions remained elusive. This team followed a traditional strategy and tested the peptides on all orphan GPCRs described by the IUPHAR that were expressed in the cells and tissues responding to the studied peptides. Neuronostatin is produced from the somatostatin preprohormone and seems to be related to cardiovascular and metabolism regulation [142]. The tissues responding to neuronostatin stimulation such as hypothalamus, heart, pancreatic α-cells, and the gastric tumor cell line KATOIII were screened to identify orphan GPCR that could bind the peptide. Four orphan GPCRs were short-listed, including GPR107 and GPR146 [141]. The specific knockdown of GPR107 impaired the expression of c-Fos, which is normally induced by neuronostatin stimulation of KATOIII cells. Moreover, the neuronostatin injection in rat lateral

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cerebroventricle induced an increase of mean arterial pressure that seems to be dependent on GPR107 expression [141]. Interestingly, neuronostatin is also implicated in the regulation of glucagon expression and secretion in pancreatic α-cells [143]. GPR107 could be responsible for this effect because it is endogenously expressed in pancreatic α-cells. In addition, when GPR107 was down regulated, stimulation with neuronostatin did not induce PKA phosphorylation and proglucagon mRNA production [144].

Following the identification of GPR107 as the putative receptor for Neuronostatin, Yosten et al. proposed GPR146 to be the orphan GPCR mediating the effect of proinsulin C-peptide [145]. The expression of GPR146 in KATOIII cells was correlated to the expression increase of c-Fos induced by specific proinsulin C-peptide stimulation. Moreover, the knockdown of GPR146 impaired the proinsulin C-peptide dependent effect on c-Fos gene expression. Internalization of GPR146 was also observed when KATOIII cells were stimulated with proinsulin C-peptide [145].

For both GPR107 and GPR146, these putative pairings could make these receptors attractive drug targets in diseases such as diabetes. For instance, proinsulin C-peptide was shown to play a role against diabetes associated complications [146]. In addition, interferon-β and interferon-γ have been shown to produce an increase of GPR146 expression suggesting a possible antiviral role for this receptor [147]. However, the same team conducted these deorphanizations and no direct evidence of binding between the ligands and the receptors was provided in the initial reports. Therefore, independent labs should perform a thorough confirmation to validate the suggested pairings.

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3.2. Problematic Ligand-receptor pairs

One of the key aspects in the orphan GPCR field is the reproducibility of the results. Some receptors have sparked controversies recently and we review here the receptors that received current attention.

FPR2/ALX - Lipoxin A_4

Formylpeptide Receptors (FPR1-3) are activated by N-formylated peptides derived from bacteria or mitochondria (except FPR3) and contribute to the migration of phagocytes to sites of infection and inflammation [148]. Lipoxin A_4 (LXA_4) is derived from arachidonic acid through the lipoxygenase pathway and has been described as an agonist for FPR2 [149]. Specific activation of the receptor by LXA_4 is though to promote a physiological resolution of inflammation. Hence, the FPR2 receptor is also referred to as ALX receptor [149]. Endogenous compounds acting as potent anti-inflammatory agents should have attracted attention as innovative therapies. However no translational research has emerged from this ligand-receptor system identified in the 90s. We, and other, failed to demonstrate any activation of FPR2/ALX by Lipoxin A_4, both in heterologous and native systems [150-152]. In addition, another team showed no evidence of FPR2/ALX activation by 15-epi-Lipoxin, a derivative supposed to act through FPR2/ALX [153], while earlier reports also showed some inconsistencies in Lipoxin A_4 signaling [154]. In a recent update, the IUPHAR Nomenclature Subcommittee for Leukotriene Receptors pointed that divergent results were present in the literature [155]. The subcommittee questioned the physical integrity of the ligands that were used by teams unable to reproduce the initial Lipoxin-FPR2/ALX pairing [155]. Although this might have been a valid explanation, it is not entirely convincing. In all negative results papers, proper controls were use, and the physical presence of intact Lipoxin A_4 at anticipated concentration was even verified by mass

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spectrometry before and after the experiments in one case [150]. Given the very potent bioactivities of Lipoxin A_4 and its therapeutic potential, a satisfactory solution such as providing standardized reagents and protocols to independent labs should be supplied by the IUPHAR in order to advance the field beyond such debate.

ChemR23 – Resolvin E1 and GPR32 - Resolvin D1

Resolvins are a recently described family of autacoids. They are synthesized by leukocytes at the site of inflammation and are believed to participate to the process of resolution of inflammation [156]. Thus, they should not be seen as anti-inflammatory but rather as a physiological process that control the magnitude and duration of normal inflammation, notably by stopping further neutrophil influx [157]. The same lab that the one involved in the discovery of the Lipoxin receptor proposed that Resolvin E1 is a ligand for ChemR23 [158]. This previously reported orphan receptor related to chemokine receptors was identified as the receptor for Chemerin [159], a pairing that is now considered as validated [22]. More recently the same team proposed that GPR32 had the ability to be activated by Resolvin D1, D3 and D5 [160,161]. However, some authors could not reproduce the activation of either ChemR23 or GPR32 by resolvins [124,162]. In their recent update, the IUPHAR Nomenclature Subcommittee for Leukotriene Receptors raised similar potential explanation than with lipoxin receptor-related discrepancies. Given the therapeutic possibilities of exploiting the resolution of inflammation, these issues should be prioritized in replication studies.

GPR17 - Cysteinyl-LT and uracil nucleotides

An involvement of GPR17 in the myelinisation process has been suggested based on its expression in oligodendrocytes and functional studies in mice [163]. Therefore, it could represent

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an attractive drug target for severe demyelinating central nervous system diseases such as multiple sclerosis. GPR17 is an orphan receptor whose ligands are debated in a very active community, given its potential interest in physiology and medicine. Based on sequence homology, GPR17 is close to both the Cysteinyl leukotrienes (Cyst-LT) and purinergic (P2Y) receptors. GPR17 was claimed to be a receptor for both Cyst-LT and uracil-nucleotides, which is somehow consistent with this phylogeny [164]. In 2010, Benned & Rosenkilde independently confirmed the activation of GPR17 by uracil-nucleotides [165]. However, a careful analysis of the report shows that critical controls are missing in the key confirmation experiment (Figure 5 of the reference [165]). The authors use a valid reporter gene assay in HEK293 cells transiently co-transfected with GPR17 and promiscuous G proteins but the results obtained with cells solely transfected transfected with promiscuous G proteins were not included in the figure. Therefore, given the high sensitivity of the reporter assay, the recorded signal may also have originated from some endogenously expressed uracil nucleotides receptors unrelated to GPR17. In addition, no independent investigators reported the detection of GPR17 activation by Cyst-LT [166].

The case of GPR17 is particularly interesting because it is an example of how surrogate ligands can clarify the function of an ill-characterized receptor. Recently, Hennen et al. identified MDL-29,951 and other 4,6-dichloro-1H-indole-2-carboxylic acid derivatives as synthetic GPR17 agonists in elegant studies [167,168]. The compound was active in a wide range of assays and permitted the clear depiction, for the first time, of a comprehensive signaling profile of the receptor when pharmacologically activated. In addition, these tools could be used to define more convincingly a function for the activated receptor in oligodendrocytes [169]. More importantly, this surrogate ligand gave access to a positive control for receptor activation and a follow up study by the same team tentatively tried to put an end to the controversy about the receptor

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ligands. In an impressive set of negative data, the authors were unable to detect any evidence of activation of the receptor by its previously assigned endogenous ligands [170]. Therefore, it seems that the quest for GPR17 endogenous agonist must continue. It should be noted that other functions of GPR17 that have been suggested in the aging brain or regulation of food intake have been partially evidenced with dubious pharmacological agents [171-173]. These data should therefore be interpreted with caution.

GPR18 - Arachidonoyl glycine

GPR18 is phylogenetically related to cannabinoid receptors and several functions for this receptor have been suggested in various diseases and systems, including intraocular pressure, metastatic melanoma or macrophage apoptosis [174-176]. Notably, it is highly expressed in lymphocytes [177]. A lipid derivative, N-arachidonoyl glycine (NAGly) has been proposed in 2006 as a ligand with an EC_{50} of 20 nM for the inhibition of forskolin-induced production of cAMP [177]. Two recent independent reports have failed to reproduce the initial pairing. Firstly, Lu et al. have used a comprehensive array of assays but failed to recapitulate the initial observations [178]. Secondly, Finlay et al. have not been able to evidence any response of the receptor by the reported ligand, although they detected an important constitutive activity [179].

GPCRC6A - Osteocalcin

GPRC6A, a class C receptor, has been reported to respond to nutrient derived factors, such as calcium and basic L-amino acids [180,181] but also testosterone [182] and the bone-derived hormone osteocalcin [183-185]. However, Rueda et al. in 2016 were unable to reproduce the results with osteocalcin, although they confirmed that the receptor could be activated by L-amino acids, notably arginine, ornithine and lysine [186]. Thus, this recent publication questions

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the interest of this receptor as a drug target in endocrine and metabolic disorders and additional studies are required to explain these discrepancies.

Other receptors

In 2013, Southern *et al.* performed a general, screening-like, study on most orphans. They used an arrestin recruitment-based assay and various libraries of endogenous ligands. Although they did not specifically addressed the issue, they could not reproduce some of already claimed pairings such as GPR3, GPR6, GPR12 (as S1P receptors), GPR23 (Lysophosphatidic acid) or GPR30 (17β-Estradiol) [124]. The other discrepancies they observed have been discussed above for GPR17, GPR18, GPR37 and GPR75.

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4. Conclusions

Past and current successes of GPCRs in terms of drug discovery have demonstrated that this family of receptors is an important source for innovative therapeutic strategies. The research groups active in the narrow orphan GPCR field are at the forefront of GPCR Drug Discovery process. Without clearly defined function, the therapeutic interest of orphans and other elusive receptors may be difficult to grasp. However, renewed creativity together with persistence on selected orphans might open avenues and lead to true groundbreaking discoveries. The present analysis demonstrates that orphan receptors remain a major untapped reservoir of innovative drug targets. Our survey of the current literature identified several issues that preclude further advances in the field.

Firstly, the number of deorphanization has decreased and remained low for the past 10 years (Fig. 1). However, there is nothing that suggests that it is because of insurmountable technical hurdles. Recently, significant advances have been made in many aspects of physiology following deorphanizations (See Table 1 and Davenport et al. [22]). Therefore, current and future research on the remaining orphans should be maintained and amplified with new innovative tools in order to maintain or even increase the current rate of deorphanization.

Secondly, we noticed a surprisingly high number of irreproducible ligand-receptor pairs along with discrepancies difficult to reconcile (see Table 2). Given the importance of proper definition of function for the remaining orphans, novel deorphanizations should be carefully assessed before claiming a new ligand-receptor pair. The reports that were later debated or unconfirmed have several points in common. Firstly, they are usually based on a single technique to assay the receptor activation. The gold standard when characterizing a new ligand is two use at least one (preferably two) orthogonal assays. These can be defined as assays demonstrating the

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same cellular effect (such as activation of a given signaling pathway) but in a completely unrelated technical environment. For instance, the detection of arrestin recruitment with a luciferase-based complementation assay should be confirmed with a system with no luciferase or even enzyme involved, such as the redistribution of signal from an arrestin fused with GFP by confocal microscopy. In addition, distinct GPCR signaling pathways should be assayed and the test should be as close as possible to the receptor activation event. From this perspective, reporter gene assays should be avoided because they are remote from receptor activation and are prone to artefacts and indirect effects due to amplification. Secondly, we noticed that in several cases there was already an important effect on non-transfected cells. Rather than normalization, the best option would be to find a system where the putative ligand has no effect on the background. When the elimination of background is technically difficult, for instance in case of ligand-receptor promiscuity, surrogate ligands represent invaluable tools as positive controls or antagonists. Another popular strategy consists in using cellular background more remote from humans and mammals such as yeast [187], to avoid the presence of endogenous receptors.

These recommendations may be useful for the future to avoid misleading reports and pairings. However, the existing literature can also hold back current research on some given orphans. Before entering into drug development or further physiological conceptual framework, these pairings should be thoroughly confirmed. While preparing this manuscript, we noticed that a significant number of receptors received no attention for the past 10 years although they have been "paired" to a ligand by a single paper. It is tempting to speculate that investigators and funders are reluctant to perform studies that may lead to "negative" data. However, although it can be quite frustrating to pursue a scientific project with high risk of generating negative data, these experiments and their description in the literature are of paramount importance. Another

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possible issue is that they might be perceived as more difficult to publish or less rewarding for career advancement. The basic pharmacology journal should promote such negative studies that could correct literature and unblock the quest on some receptors. In addition, inexperienced teams are susceptible to start project based on the assumption that some mediators work through a given receptor, only to realize at later stage that the starting hypothesis was not robust enough. Therefore, effort in reproducing independently initial pairing could spare a significant amount of valuable time and resources. It should be noted that there seem to be an encouraging trend as several important "negative" reports have been published recently, on GPR18 or GPR17 for instance (Table 2).

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6. Conflict of interest

The authors declare no conflicts of interest.

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Figure and Table legends

Figure 1. Number of reported GPCR deorphanization in the literature between 2000 and 2017 (Adapted from Levoye & Jockers [28]).

Table 1. Orphan GPCR recently reported as being deorphanized (2013-2017).

Table 2. Problematic ligand-receptor pairs with inconsistencies that have been recently investigated.

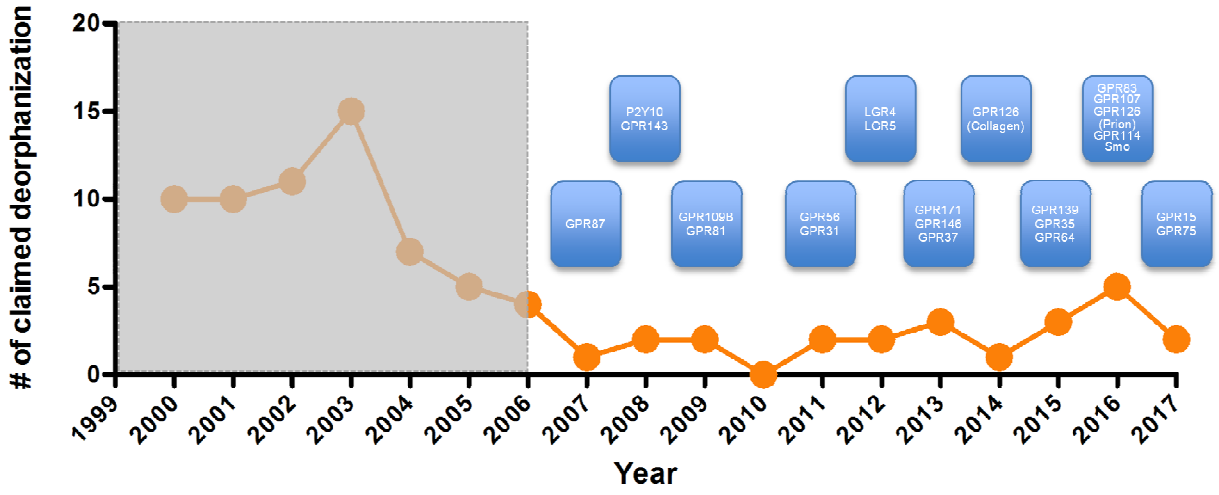

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Table 1. Orphan GPCR recently reported as being deorphanized (2013-2017).

Receptor	New ligand	Reference
GPR15	GPR15L	[79]
GPR35	CXCL17	[88]
GPR37	Prosaposin and prosaptide	[125]
GPR64	Tethered peptide agonist- " Stachel sequence"	[133]
GPR75	20-Hydroxyeicosatetraenoic acid (20-HETE)	[139]
GPR83	PEN	[106]
GPR107	Neuronostatin	[141]
GPR114	Tethered peptide agonist- " Stachel sequence"	[134]
GPR126	Type IV Collagen Tethered peptide agonist- " Stachel sequence" Prion protein PrP(C)	 [130] [136]
GPR133	Tethered peptide agonist- " Stachel sequence"	[130]
GPR139	L-tryptophan and I-phenylalanine Adrenocorticotropic hormone α-melanocyte stimulating hormone β-melanocyte stimulating	[93,94] [98]

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	hormone The conserved core motif HFRW	
GPR146	Proinsulin C-peptide	[145]
GPR171	Big LEN	[105]

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Table 2. Problematic ligand-receptor pairs with inconsistencies that have been recently investigated.

Receptor	Ligand	Initial pairing	Inconsistencies in
FPR2	Lipoxin A4	[188]	[150-152]
GPR32	Resolvin D1	[160]	[124]
GPR1/ChemR23	Resolvin E1	[158]	[189]
GPR18	N-arachidonoylglycine	[177]	[179]
GPR17	Uracil Nucleotides & Cysteinyl Leukotrienes	[164]	[170]
GPR37	Prosaposin and prosaptide Head activator peptide	[125]	[119,124]
GPRC6A	Osteocalcin	[183]	[186]

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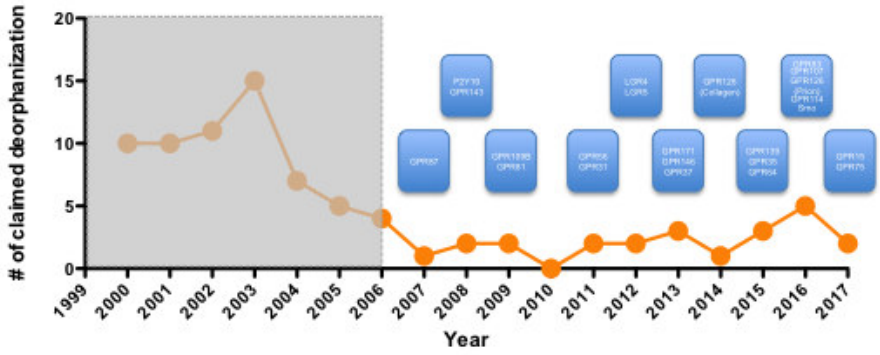
Claimed deorphanizations during the 2007-2017 period



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