Minireview

Deorphanization of G Protein Coupled Receptors (GPCRs): a historical perspective.

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Running title: GPCR deorphanization

Manuscript Pages: 30
Manuscript Figures: 1
Manuscript Tables: 1
Abstract Word Count: 170
Introduction Word Count: 860
Discussion Word Count: 6024

Abbreviations:

2-AG - 2-Arachidonoyl-Glycerol
5-HT - Serotonin or 5-hydroxytryptamine
7TM - 7 transmembrane
AEA - Arachidonoyl-Ethanolamine
APLN - Apelin
APLNR - Apelin Receptor
CB1R - Cannabinoid Receptor 1
CB2R - Cannabinoid Receptor 2
CBD - Cannabidiol
CBN - Cannabinol
CMKLR1 - Chemokine-like Receptor 1
CNS - Central Nervous System
DMR - Dynamic Mass Redistribution
GABA - γ-Aminobutyric Acid
GH - Growth Hormone
GHRH - Growth Hormone-Releasing Hormone
GHRP - Growth Hormone-Releasing Peptide
GHS - Growth Hormone Secretagogue
GPCR - G Protein Coupled Receptor
NC-IUPHAR - Nomenclature and Standards Committee of the International Union of Basic and Clinical Pharmacology
PKC - Protein Kinase C
PTX - Pertussis Toxin
THC - (-)-Δ9-tetrahydrocannabinol
VFT - Venus-flytrap module
Abstract

Counting over 800 members, G Protein Coupled Receptors (GPCRs) form the largest family of membrane receptors encoded in the human genome. Since the discovery of G proteins and GPCRs in the late 1970s and early 1980s, a significant portion of the GPCR research has been focused on identifying ligand/receptor pairs in parallel to studies related to their signaling properties. Despite significant advancements, about a fourth of the ~400 non-odorant GPCRs are still considered orphan because their natural or endogenous ligands have yet to be identified. We should consider that every GPCR was once an orphan and that endogenous ligands have often been associated with biological effects without a complete understanding of the molecular identity of their target receptors. Within this framework, this review offers a historical perspective on deorphanization processes for representative GPCRs, including Ghrelin receptor, GABAB receptor, Apelin receptor, Cannabinoid receptors, and GPR15. It explores three main scenarios encountered in deorphanization efforts and discusses key questions and methodologies employed in elucidating ligand-receptor interactions, providing insights for future research endeavors.

SIGNIFICANCE STATEMENT

Understanding how scientists have historically approached the issue of GPCR deorphanization and pairing of biologically active ligands with their cognate receptors are relevant topics in pharmacology. In fact, the biology of each GPCR, including their pathophysiological involvement, has often been uncovered only after their deorphanization, illuminating druggable targets for various diseases. Furthermore, uncovered endogenous ligands have therapeutic value as many ligands - or derivates thereof - are developed into drugs.

Introduction

G protein coupled receptors (GPCRs) are 7 transmembrane (7TM) proteins and form the largest family of receptors in the human genome. GPCRs play a pivotal role in mediating the effects of a wide range of extracellular signals, including neurotransmitters, hormones, and sensory stimuli. They are classified according to sequence homology into classes, with class A (rhodopsin-like) being the most numerous (www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=694&familyType=GPCR). Currently, almost 100 GPCRs are classified as “orphans” because their natural/endogenous ligands and often their
signaling pathways are unknown (Harding et al., 2024). We refer to GPCR deorphanization as the process that leads to the definitive pairing of endogenous/natural ligands to the activation of a specific GPCR or GPCR family. Upon deorphanization, the orphan receptor name is changed from “GPR” to a name that reflects its endogenous ligand, and the name is chosen by a committee from the Nomenclature and Standards Committee of the International Union of Basic and Clinical Pharmacology (NC-IUPHAR; www.guidetopharmacology.org/nomenclature.jsp). Historically, the process of GPCR deorphanization has been a critical aspect of understanding their functions and implications in various physiological and pathological processes.

In the early '90s, homology-based cloning methods allowed scientists to identify novel members of a multigene family by using degenerate primers partially overlapping with the few known GPCR coding sequences. Using such approaches, it was predicted that hundreds of GPCRs would serve as odorant receptors (Buck and Axel, 1991). Later on, the completion of the Human Genome Project in 2001 (Lander et al., 2001; Venter et al., 2001), combined with the prediction of transmembrane domains in the encoded genes, allowed the recognition of over 800 proteins exhibiting the characteristic 7TM structure. It was then confirmed that half of these receptors are mostly expressed in the olfactory system where they mediate the sense of smell (Gaillard et al., 2004). The remaining ~400 GPCRs are expressed throughout tissues and organs with exclusive patterns of cell specificity. Their restricted landscape of expression creates a first layer of complexity that allows GPCRs to convert a variety of extracellular stimuli into tissue- and cell-specific physiological responses. Homology cloning techniques are based on the assumption that novel GPCRs would share a high homology of sequence with the known GPCR used to design degenerate primers. Successfully, using monoaminergic GPCRs as a template led to the cloning of multiple epinephrine/norepinephrine, serotonin, and histamine receptors (Dixon et al., 1986; Fujiwara et al., 1990; Gantz et al., 1991; Libert et al., 1989; Pritchett et al., 1988; Yamashita et al., 1991). However, high homology between receptors does not always implicate that their activation is driven by the same or similar ligands. As an example, GPR1 was originally cloned exploiting its sequence homology to the δ-opioid receptor gene (Marchese et al., 1994). However, it was later shown that GPR1 does not bind opioid ligands while it is more closely related to Chemokine-like Receptor 1 (CMKLR1) (Meder et al., 2003; Wittamer et al., 2003), and binds the CMKLR1 endogenous agonist chemerin (Barnea et al., 2008).

Traditionally, endogenous GPCR ligands have been identified in specific fractions or extracts from tissues and organs. Evidence for the presence of active compounds in the sample was produced by functional
assays that measured unique physiological responses of the organ/tissues, i.e. contraction/relaxation of cardiac muscles, womb, bladder, etc. Well-known examples are serotonin (5-hydroxytryptamine; 5-HT) isolated from salivary glands of *Octopus vulgaris* through chromatography (Erspamer and Boretti, 1950); or enkephalins isolated from pig brains by multistep fractionation methods followed by mass spectrometry, which were then tested in dose-response experiments against naloxone assessing relaxation levels of guinea pig ileum and mouse vas deferens (Hughes et al., 1975a; Hughes et al., 1975b). A number of methodological approaches have been developed and applied over the years to pair GPCRs and their ligands. This review is not intended to go into detail about these strategies which have already been described in length elsewhere (Chung et al., 2008; Fang et al., 2008; Franchini and Orlandi, 2023; Jobe and Vijayan, 2024; Satake et al., 2023; Yasi et al., 2020).

In the present review, we offer a brief historical overview of the process of pairing endogenous ligands with their cognate GPCRs for a selection of representative receptors including Ghrelin receptor, GABA<sub>B</sub> receptor, Apelin receptor, Cannabinoid receptors, and GPR15. Our goal is to offer novel insights and conceptual frameworks that may prove beneficial for future receptor deorphanization efforts. We will address the three predominant scenarios encountered by researchers (Figure 1): 1. an endogenous molecule, an orphan ligand, was originally proposed as a putative ligand for an undiscovered GPCR (i.e. GABA); 2. a synthetic or natural compound was initially suggested as acting on an unknown GPCR (i.e. cannabinoids, ghrelin); or 3. a GPCR was identified and characterized for its physiological function, but its endogenous ligand was unknown (i.e. GPR15, APJ receptor). Milestones in the discovery process related to the discussed GPCRs are listed in Table 1. We will discuss key questions such as the nature of the observed original physiological effects, the rationale for the hypothesis that a GPCR mediates said effects, the methodologies employed for receptor identification of known ligands, or vice versa, to determine the ligand activating a known receptor, and, finally, the insights gained from this investigative process.

**Ghrelin Receptor**

The pairing of the gastrointestinal peptide hormone ghrelin with its cognate receptor GSH-R1 followed an unusual path (reviewed in (Bowers, 2012)). In the early 1980s, Bowers and Momany developed synthetic opioids based on the amino acid sequence of met-enkephalin that stimulated the release of growth hormone (GH) from the anterior pituitary and were thus referred to as GH releasing peptides (GHRPs) (Bowers, 2012; Bowers et al., 1980; Momany et al., 1981). Initially believed to act solely on the pituitary, it was later
discovered that these peptides also prompted GH release from the hypothalamic arcuate nucleus acting on a receptor different from the one activated by the growth hormone-releasing hormone (GHRH) (Dickson et al., 1993; Gaylinn et al., 1993; Lin et al., 1992; Mayo, 1992). At the cellular level, GHRPs and synthetic GH secretagogue compounds (GHSs) inhibited potassium channels, prompted transient intracellular calcium mobilization, increased intracellular concentrations of inositol trisphosphate and produced subsequent activation of protein kinase C (PKC) (Bresson-Bepoldin and Dufy-Barbe, 1994; Cheng et al., 1991; Herrington and Hille, 1994; Lei et al., 1995). Altogether, these are cellular effects that can be attributed to the activation of an unknown GPCR. In 1996, employing the GHS compound MK0677, it was possible to clone the GH secretagogue receptor (GHS-R1), revealing that GHRPs and GHSs were agonists at this GPCR (Howard et al., 1996). Specifically, Xenopus oocytes were injected with cRNA from swine pituitary and then treated with MK0677. Calcium mobilization responses were detected using a co-injected biosensor or measuring oscillatory increases in calcium-activated chloride currents. Co-expression of Gα11 helped in the first stages to improve the signal obtained when injecting complex cRNA pools, but was not required after fractionation and enrichment in cRNA expressing the pursued GPCR. Stepwise fractionation resulted in the identification of a single clone encoding a 353 amino acid protein containing 7TM domains: GHS-R1a. Radioligand binding experiments confirmed a specific interaction of MK0677 with GHS-R1a that was not observed with other endogenous peptides acting on GPCRs such as galanin, GHRH, GnRH, CRF, neuromedin B, and TRH (Howard et al., 1996). Soon after, GSH-R1a expression was mapped in the rat brain as enriched in hypothalamic nuclei, pituitary gland, and dentate gyrus of the hippocampus (Guan et al., 1997), and later in peripheral tissues including pancreatic islets, adrenal gland, thyroid, and myocardium (Gnanapavan et al., 2002). These findings suggested that GSH-R1a controls a variety of physiological processes in addition to GH release (Muller et al., 2015).

After establishing that GHS-R1 was the GPCR responsible for mediating the effects of these synthetic compounds and peptides, the search for its endogenous ligand continued for three more years. To this goal, Kojima and colleagues established a CHO cell line stably expressing rat GSH-R1 as a tool to monitor changes in the intracellular calcium concentrations in response to application of rat tissue extracts from brain, lung, heart, kidney, stomach, and intestine (Kojima et al., 1999). Stomach extracts revealed the highest biological activity and peptides were further purified. In detail, they treated rat stomach to block protease activity and proceeded to homogenize the tissue. The resulting extract was then further processed to obtain fractions which were then applied to the stable CHO cells to test intracellular calcium mobilization.
Active fractions were then separated by consecutive steps of HPLC to obtain a pure peptide that was eventually sequenced. The identified endogenous peptide agonist was named ghrelin: a 28-amino acid hormone produced by the gastric oxyntic cells (Kojima et al., 1999). Ghrelin was tested in different functional assays, establishing its ability to stimulate the release of different hormones, including GH, from pituitary cells. Its ability to increase Ca$^{2+}$ concentrations in the CHO cell line stably expressing GSH-R1 was abolished by treatments with an antagonist (Kojima et al., 1999). Further studies on ghrelin and its receptor GHS-R1a revealed multiple roles in feeding behavior and energy homeostasis, adiposity, and glucose metabolism. Additionally, peripheral functions were described including stimulation of gut motility and gastric acid secretion, modulation of sleep, taste sensation and reward seeking behavior, modulation of stress and anxiety, protection against muscle atrophy, and improvement of cardiovascular functions (Muller et al., 2015; Tschop et al., 2000; Wren et al., 2001).

We can infer from the deorphanization process of the ghrelin receptor that some biological functions are governed by multiple mechanisms (see for example the release of GH). As a result, substances that have the same biological effects may act through completely different receptors. The vasodilation effect is a prime illustration of this idea. It is facilitated by various drug classes that function through distinct molecular and cellular mechanisms, including phosphodiesterase inhibitors (sildenafil, tadalafil), direct-acting vasodilators (nitroprusside, nitrates), β2-adrenergic agonists (salbutamol, salmeterol), calcium-channel blockers (verapamil, diltiazem, nifedipine), and others. We also recognize the significance of the molecular characterization of receptor signaling, which enables researchers to distinguish between the various receptors involved and their respective roles in the process. In the course of discovering GH-releasing hormones and their receptors, scientists used extracts from various tissues and organs to confirm the presence of GH-releasing compounds. However, due to the simultaneous presence of somatostatin in the hypothalamus, which inhibits the release of GH from the pituitary and counteracts both GHRH and GHRP activity, hypothalamic extracts applied to pituitary glands were unable to produce a significant GH release. Therefore, to prevent false-negative results, special attention should be paid to the concentration of endogenous ligands and the existence of possible counteracting compounds in organ extracts.

γ-Aminobutyric Acid (GABA) receptors
The identification of γ-Aminobutyric Acid (GABA) as a novel neurotransmitter was first reported by Roberts and Frankel in 1950, who noted a very abundant compound in brain extracts from different species, but not in other organs or tissues. Using layer chromatography, they isolated an unknown compound from brain extracts that chemically behaved similarly to histidine, methionine-sulfoxide, and GABA (Roberts and Frankel, 1950). Further chromatographic experiments confirmed the identity of the compound as GABA and in the following years, several labs confirmed the presence of GABA in the brain (Awapara et al., 1950; Udenfriend, 1950). In detail, Awapara and colleagues extracted GABA from bovine brain. More in detail, the brain homogenate was processed with several steps of extractions and centrifugations till layer chromatography finally led to the identification of GABA. In vivo, GABA is obtained by decarboxylation of glutamic acid and it was initially studied mostly for its role in the maintenance of glutamate levels in the tricarboxylic acid cycle. Nevertheless, in 1959, Curtis and colleagues characterized the excitatory and inhibitory pharmacological profiles of amino acids and structurally related metabolites on neurons, discovering that glutamic acid, aspartic acid, and cysteic acid are the main excitatory amino acids in the nervous system; while their decarboxylated forms such as GABA, β-alanine, and taurine have inhibitory roles (Curtis et al., 1959). Further studies revealed an inhibitory action of GABA on neuronal excitability by reducing epileptic events in dogs, and by reducing impulse transmission on neuromuscular junction of crayfish (Basemore et al., 1957; Hayashi, 1959). These results clearly indicated an inhibitory function of GABA on neurotransmission and the possibility that GABA could represent a novel neurotransmitter. The existence of membrane components mediating the effects of neurotransmitters was only a hypothesis at the time. The use of radiolabeled GABA analogues allowed to localize GABA receptors in the central nervous system (CNS), mostly in the cerebellar granular layer, and in sympathetic ganglia (Bowery and Brown, 1974; Wilkin et al., 1981; Zukin et al., 1974). Significantly, this binding was prevented by application of bicuculline, a convulsant agent previously shown to act as an antagonist of a yet unidentified GABA receptor (Curtis et al., 1970; Curtis et al., 1971; Straughan et al., 1971). The observation that GABA receptors localized on cell bodies were responsible for reduced spike amplitude and slow conduction by increasing chloride conductance, and they were blocked by bicuculline, while axon terminal GABA receptors were responsible for a reduction in neurotransmitter release, and were insensitive to bicuculline, suggested these effects were likely produced by the activation of different types of GABA receptors (Brown et al., 1979; Brown and Higgins, 1979). Soon later, this hypothesis was further supported by the development of baclofen, a GABA analogue which was inactive at bicuculline sensitive sites but was as active as GABA in reducing evoked neurotransmitter release, reinforcing the idea of two different GABA receptors named GABA_A and GABA_B.
(Bowery et al., 1980; Hill and Bowery, 1981). Because of the rapid increase in post-synaptic chloride conductance, it was clear that the GABA<sub>A</sub> receptor complex was likely a GABA-regulated ion channel composed of multiple subunits (Olsen, 1982). This was soon confirmed when the GABA<sub>A</sub> receptor was successfully affinity purified from bovine brain (Sigel and Barnard, 1984; Sigel et al., 1983) and later cloned (Schofield et al., 1987).

For the first time in 1989, GABA<sub>B</sub> was described as a metabotropic receptor (Holz et al., 1989). Pioneering studies demonstrated that GABA could inhibit voltage-gated calcium channels in peripheral sensory neurons from embryonic chick dorsal root ganglia (Dunlap and Fischbach, 1981). Using this model, it was later found that GABA-mediated effects could be blocked by the pre-incubation of neurons with pertussis toxin (PTX), which blocks signaling mediated by heterotrimeric G<sub>proteins, or by pre-incubation with a GDP analogue (GDP-β-S), a non-hydrolyzable analogue of GDP that competitively inhibits the binding of GTP to G proteins, overall indicating that GABA<sub>B</sub> was a G<sub>proteins-coupled GPCR (Cassel et al., 1979; Holz et al., 1986; Katada et al., 1984). In 1997, Kaupmann and colleagues were finally able to identify the GABA<sub>B</sub> receptor components and sequence them. To this aim, they developed [{sup 125}I]CGP71872, a photoactivatable radiolabeled GABA<sub>B</sub> receptor antagonist with nanomolar affinity able to be selectively cross-linked to GABA<sub>B</sub> receptor (Kaupmann et al., 1997). Photo-crosslink analysis of [{sup 125}I]CGP71872 with several tissue extracts revealed two N-glycosylated proteins of 100 and 130 KDa that showed predominant expression in the CNS. Given the high expression of GABA<sub>B</sub>R in cortex and cerebellum of rat brain (Turgeon and Albin, 1994), a library of cDNAs was obtained from these regions and transfected into COS1 cells. A subsequent screening for radiolabel binding with [{sup 125}I]CGP71872 was performed with serial subdivisions until only one positive cDNA was identified. A cDNA encoding a protein of approximately 130 KDa, GABA<sub>B</sub>R1a was isolated from this first cDNA library screening. Then, a more stringent screening was performed using cDNA from GABA<sub>B</sub>R1a isolating a cDNA encoding a 100 KDa protein, GABA<sub>B</sub>R1b, characterized by a shorter extracellular N-terminus. Amino acid sequence analysis of the cloned receptors revealed that the metabotropic receptors for GABA and glutamate belong to the same class of GPCRs. Further binding studies using cells expressing the newly cloned GABA<sub>B</sub>R1a-b confirmed the identity of these proteins as the targets of available agonists and antagonists. Finally, a pharmacological characterization was performed using adenylyl cyclase inhibition in transfected cells as an index of GABA<sub>B</sub> receptor activation. Notably, prominent differences were observed in terms of ligand potency in activating native GABA<sub>B</sub> receptors in tissues and in cells transfected with the cloned GABA<sub>B</sub>R1 (Kaupmann et al., 1997). These differences led to hypothesize the existence of different
GABA₂ subtypes. Follow-up studies used the sequence of the identified GABA₉R1 to isolate similar GPCRs leading to the discovery of GABA₉R2 (Kaupmann et al., 1998). Three independent groups simultaneously recognized how the functional GABA₉ receptor is an obligate heterodimer of GABA₉₁ and GABA₉₂ receptors (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998). In these studies, analysis of cells co-expressing both monomers revealed agonist potencies similar to what was previously observed in native tissues. More recently, cryo-EM structures of multiple active and inactive states revealed further details of GABA₉ receptor activation (Papasergi-Scott et al., 2020; Park et al., 2020; Shaye et al., 2020). Both subunits display a venus-flytrap module (VFT) in the extracellular N-terminal region followed by a linker to the 7 TM domains. Despite both subunits contain the VFT module representing the ligand-binding domain for class C GPCRs, it was found that only the VFT of GABA₉₁ interacts with the neurotransmitter GABA (Kniazeff et al., 2002). Studies on GABA₉₂ subunit revealed this component is responsible for signaling properties of the receptor and membrane targeting of the heterodimer (Burmakina et al., 2014; Galvez et al., 2001; Margeta-Mitrovic et al., 2000). As expected by original studies using PTX studies, stimulation of GABA₉R triggers activation of Ga proteins of the Gi/o/z subfamily initiating signaling cascades responsible for the above reported physiological effects (Bettler and Tiao, 2006).

From the early discovery of GABA as a neurotransmitter to the recognition of the existence of both ionotropic and metabotropic receptors activated by GABA, to the final identification of two GPCR monomers that act as an obligate heterodimer, we can appreciate how the development of novel technologies was critical in each stage of the pairing of GABA to its receptors. For example, the discovery of high-affinity antagonists and their development into photoactivatable radiolabeled compounds was crucial in the identification of GABA₉ receptors. More recently, an analogous method was employed to deorphanize GPRC5A (Zhao et al., 2023), another class C orphan GPCR that has been receiving more attention for its role in cancer biology, inflammation, and immunity (Iglesias Gonzalez et al., 2023). The authors of this study looked at aromatic chemicals derived from microbiota that have been proposed as potential GPCR ligands. They specifically derivatized indole-3-acetic acid (IAA), a metabolite that is frequently produced by the microbiota, using a photo-affinity moiety for UV crosslinking and bio-orthogonal tags for detection and affinity enrichment. These compounds were subsequently employed as baits to separate IAA-interacting proteins from intestinal epithelial cells that were later identified by mass spectrometry. Applying this method, GPRC5A and a few other GPCRs were found to be potential IAA receptors (Zhao et al., 2023). Lastly, high-order complexes of orphan GPCRs are a possibility suggested by the obligate heterodimerization of GABA₉₁ and GABA₉₂.
receptors, which indicates that deorphanization efforts should take this additional factor into account. This is especially the case for class C GPCRs (Ellaithy et al., 2020; Fu et al., 2022; Jeong et al., 2021; McCullock and Kammermeier, 2021; Patil et al., 2022; Shin et al., 2024). Finally, similar to how GABA was considered an "orphan ligand" until the molecular characterization of its membrane receptors, many other bioactive substances have been discovered and functionally defined. It is reasonable to expect that some of these orphan ligands, which include peptides, lipids, and small molecules, will be paired with the activation of orphan GPCRs in the future.

**Apelin/Elabela Receptor (APJ receptor)**

APJ receptor (APLNR/AGTRL1/APJ) is a 380 amino acid class A GPCR cloned in 1993 because of its sequence similarity with the angiotensin receptor (O'Dowd et al., 1993). However, APJ showed no affinity for angiotensin-II, therefore it was initially classified as an orphan (O'Dowd et al., 1993). After the discovery of APJ, the analysis of its physiological role was halted by a lack of pharmacological tools to modulate its activity. Later work showed that APJ is expressed across a variety of tissues and organs, including the cardiovascular system, lung, kidney, pancreas, and brain with enrichment in hypothalamus, hippocampus, and spinal cord suggesting its involvement in multifaceted physiological responses (Hosoya et al., 2000; Lee et al., 2000; Medhurst et al., 2003; O'Carroll et al., 2000).

Five years after the discovery of APJ, its endogenous ligand was identified through steps of purification from bovine stomach extracts: a peptidic compound named apelin (APLN) (Tatemoto et al., 1998). The process of apelin identification as the endogenous ligand involved the generation of CHO cells stably expressing APJ. An increase in the rate of extracellular acidification by proton excretion, measured with a Cytosensor microphysiometer, was the method used to monitor APJ activation. This effect is in fact observed when second messengers are produced and it has the great advantage of detecting GPCR activation without previous knowledge of the coupled signaling pathway (McConnell et al., 1992). Cells were treated with concentrated peptide preparations obtained from various porcine and bovine tissues. Such preliminary studies indicated the presence of a functional endogenous ligand in bovine stomach extracts. To define the identity of this endogenous ligand, stomach tissue was homogenized, and peptides were extracted in acidic conditions. The resulting stomach extract was then tested for its ability to acidify the extracellular solution when applied to APJ-expressing CHO cells. Cells not expressing APJ served as negative controls. This
acidification-rate-promoting activity was essential in establishing the presence of a functional ligand during each step of the peptide purification and in recognizing activity-containing fractions after HPLC. The N-terminal amino acid sequence of the active purified peptide was determined as derived from the enzymatic processing of pre-proapelin, a conserved 77 amino acid polypeptide with the C-terminal 23 residues being identical in mammals. APJ was then renamed apelin receptor (APLNR). Alternative processing of proapelin gives rise to different apelin fragments, which all result in the activation of APLNR, such as apelin-36, apelin-17, and apelin-13 (Couvineau et al., 2020). Subsequent studies attributed apelin key roles in early stages of gastrulation in heart development, with apelin being one of the earliest secreted peptides during development (D’Aniello et al., 2009). Most of the physiological effects of APLNR activation have been attributed to G protein activation, in particular of members of the G_{i/o} (Masri et al., 2002) and G_{q/11} (Szokodi et al., 2002) families; however, APLNR also seems to act as a mechanosensor in cardiac tissue in a G protein-independent manner (Scimia et al., 2012).

As is the case with many peptidergic GPCRs, multiple endogenous ligands can activate one receptor or receptor family (Foster et al., 2019). Unsurprisingly, this was also true for APLNR. It was previously established that significant cardiac and vascular abnormalities resulted in 50% embryonic lethality in APLNR-KO mice (Charo et al., 2009; Kang et al., 2013). Conversely, mice that had the apelin ligand gene knocked out were born healthy and showed no sign of cardiovascular disease despite impaired heart contractility was observed with aging (Kuba et al., 2007). This inconsistency suggested that another endogenous APJ ligand was produced during embryonic development. Fifteen years after the discovery of apelin, two independent groups working at uncovering signaling axis responsible for zebrafish embryogenesis discovered a novel APJ peptide ligand that they named toddler or elabela (Chng et al., 2013; Pauli et al., 2014). In both studies elabela/toddler was identified as an endogenous agonist of APJ based on the similarity of observed phenotypes in zebrafish induced by knocking out the gene encoding this peptide and the one encoding APJ. Interestingly, this 32-amino acid peptide is produced from the cleavage of a longer pro-peptide encoded within a genome region believed to be non-coding (Chng et al., 2013; Perjes et al., 2016). Toddler/Elabela is conserved in vertebrates, expressed during embryogenesis before apelin reaches detectable levels, and downregulated in late cell developmental stages (Miura et al., 2004). Following up on the initial binding studies that proposed toddler/elabela as a novel endogenous ligand for APLNR, functional analysis confirmed the ligand/receptor pairing (Chng et al., 2013; Wang et al., 2015).
As described for apelin/APJ, the identification of receptor/ligand pairs has historically benefited from the use of tissue extracts as a source of endogenous ligands. More receptors will probably be deorphanized in the future by applying this strategy, especially in light of the constant development and improvement of new, incredibly sensitive readout techniques. The use of a minimal amount of tissue is one benefit of measuring outputs with an optimal signal-to-noise ratio. For example, allows to investigate whether ligands are present in small organs like glands or small populations of secreting cells. Furthermore, we are now aware that a large number of GPCRs can be activated by multiple endogenous agonists. In this context, the story of APLNR deorphanization shows that key information can be obtained by comparing the physiological and behavioral consequences of ablating an endogenous agonist versus its receptor. The Cytosensor microphysiometer (Hafner, 2000) was widely adopted in the late 90s to measure metabolic changes and was particularly relevant for APJ deorphanization. This technology provides a quantitative measurement of extracellular acidification rate elicited by small molecules, neuropeptides, and hormones in response to GPCR activation but also to ligand-gated ion channels and receptor tyrosine kinases (Barrett et al., 1997; Lang et al., 2001; Merkouris et al., 1997; Ng et al., 2001; Pitchford et al., 1995; Pitchford et al., 1997). Further improvements enabled simultaneous measurement of various analytes and metabolic parameters (Eklund et al., 2004); however, the Cytosensor system was not designed for high-throughput screening. The main advantage of the Cytosensor for GPCR deorphanization was its label-free nature; a concept later developed into high-throughput screening platforms that were independent from the activation of unique transducers (heterotrimeric G proteins or β-arrestins) such as Dynamic Mass Redistribution (DMR) and bio-impedance methods (Franchini and Orlandi, 2023).

**Cannabinoid Receptor System**

Consumption of Cannabis extracts and derivatives traces back more than 5,000 years, marked by their application in traditional medicinal practices, religious rituals, and recreational pursuits, particularly prized for their psychoactive attributes leading to CNS depression and analgesia (Dewey, 1986). The active components of such heterogeneous extracts are termed phytocannabinoids and are characterized by variable amounts and distinctive pharmacological profiles making it difficult to use these extracts for therapeutic purposes. Nonetheless, advancements in cannabinoid research, encompassing the synthesis of analogues, characterization of their pharmacological attributes, and exploration of biological activities, have facilitated the approval of numerous cannabinoid-derived molecules for therapeutic applications (Pagano et
Notably, among the most extensively studied phytocannabinoids are (-)-Δ9-tetrahydrocannabinol (THC), cannabidiol (CBD), and cannabinol (CBN). Historically, a first set of experiments performed by Haagen-Smit and colleagues in 1940 showed how a Cannabis extract could induce catalepsy and impair motor coordination in dogs (Haagen-Smit et al., 1940). Early studies also evidenced relatively high lethal doses and low toxicity for several phytocannabinoids when administered to mice, rabbits, guinea pigs, cats, and dogs. At the same time, the only effect common to every tested species was catalepsy (Loewe, 1946).

Additionally, (-)-Δ9-THC demonstrated a strong interaction with synthetic membranes made of phospholipids and cholesterol which did not occur with the non-psychoactive enantiomer (+)-Δ9-THC. As a result, some of the biological effects of cannabinoids were originally attributed to a non-specific disruption of cellular membranes rather than their functional interaction with a membrane receptor (Pertwee, 1988). However, experiments performed on neuronal cultures with psychoactive cannabinoids resulted in the inhibition of adenylyl cyclase and reduction in cAMP levels which was eliminated by pretreatments with PTX, indicating that a G<sub>i/o</sub>-coupled receptor was mediating their biological effects (Howlett and Fleming, 1984; Howlett et al., 1986). As a result, the “altered membrane fluidity” hypothesis was discarded.

In 1990, Matsuda and colleagues successfully cloned the first cannabinoid receptor starting from a cDNA library obtained from rat cerebral cortex (Matsuda et al., 1990). Here, using an oligonucleotide probe derived from the sequence of bovine substance K receptor, they cloned a cDNA encoding a 7 transmembrane domain protein, indicating features of a GPCR which they named SKR6. Radiolabeled binding assays and cAMP measurements with several known hormones and neurotransmitters did not reveal any conclusive activation of SKR6. Interestingly, SKR6 mRNA was absent in CHO cells, but expressed in N18TG-2 and NG108-15 cell lines. Treatments with (-)-Δ9-THC and CP55940, a cannabinoid analogue, were shown to reduce cAMP in these cell lines, indicating the co-expression of cannabinoid receptors. Similarly, cannabinoid receptors and SKR6 displayed similar anatomical distribution in the brain. Stable CHO cells expressing SKR6 revealed that treatments with cannabinoids inhibited forskolin-stimulated accumulation of cAMP in a dose-dependent manner, confirming SKR6 as the first cannabinoid receptor, later named CB1R.

Three years later, Munro and colleagues identified a novel cDNA from HL-60 cell line encoding a GPCR showing 44% identity with CB1R (Munro et al., 1993). Thus, they measured the ability of WIN5512-2, a synthetic cannabinoid receptor agonist, and other cannabinoids including CBN, CBD, and (-)-Δ9-THC to bind membranes from cells transfected with this cDNA demonstrating a different affinity profile to the new receptor compared to CB1R. Notably, while CB1R expression was restricted to the CNS (Mailleux and
Vanderhaeghen, 1992; Matsuda et al., 1990), expression of the novel cannabinoid receptor, named CB2R, was mostly observed in peripheral tissues (Munro et al., 1993).

Nearly concurrently with the discovery of a novel cannabinoid receptor was the identification of their endogenous ligands. To this goal, a clear definition of the pharmacophore groups in the chemical structure of cannabinoil allowed to develop derivatives preserving the same pharmacological profile but improving stability, pharmacokinetic, and interaction with the target. Cannabinoil was used as a lead compound to design a new agonist, HU-243, that was further developed to be used in radioligand competition assays. With this tool, Devane and colleagues set out to identify endogenous ligands for cannabinoid receptors (Devane et al., 1992). To this goal, they homogenized porcine brains in organic solvents like chloroform or methanol, centrifuged and subjected the supernatant to fractionation on a silica column. After different elution schemes, they analyzed each fraction for their ability to displace \([^{3}H]\)-HU-243 from rat synaptosomal membranes. Interestingly, they noted that the radiolabeled compound was able not only to bind specifically CB1R on synaptosomal membranes, but also non-specifically to the microfuge tube walls. Therefore, in analyzing the assay results, they paid particular attention to those fractions able to displace the compound from the synaptosomes, but not from the microfuge walls, which would otherwise indicate a false positive result. After analysis of the fractions, a further chromatography with low and medium pressure followed by a thin layer chromatography led to the isolation of a compound, arachidonoyl-ethanolamine (AEA) that they renamed anandamide (Devane et al., 1992). AEA showed a dose-dependent inhibition of twitch in murine vas deferens, a previously reported functional effect of psychotropic cannabinoids (Pertwee et al., 1992). A few years later, the same group investigated the existence of peripheral ligands activating cannabinoid receptors. These efforts led to the identification of 2-arachidonoyl-glycerol (2-AG) another endogenous ligand for CB1R and CB2R (Mechoulam et al., 1995). This time, Mechoulam and colleagues isolated lipids from canine intestine through methanol and acetone extraction. The fractions obtained were tested for radiolabeling inhibition of \([^{3}H]\)-HU-243 on synaptosomal membranes from rat brain as they have previously done with AEA. These results demonstrated that 2-AG was a novel, peripheral endogenous agonist of CB1R and CB2R (Mechoulam et al., 1995). More recent characterizations revealed that CB1R is mostly localized in the brain and skeletal muscle, with shorter splicing isoforms expressed in liver and pancreatic islets (Devane et al., 1988; Gonzalez-Mariscal et al., 2016), while CB2R is expressed mostly in immune cells, gastrointestinal tract, testis, and at low levels in the brain (Galiazza et al., 2018; Liu et al., 2009; Nunez et al., 2004). Pharmacologically, AEA displays higher affinity for CB1R compared to 2-AG and it has been
described as a CB1R partial agonist, while 2-AG has been reported to behave as a full agonist at both CB1R and CB2R (Mackie et al., 1993; McAllister et al., 1999; Savinainen et al., 2001; Sugiura et al., 1999). Since the identification of cannabinoid receptors, several other endogenous arachidonoyl derivatives has been proposed, including for example N-arachidonoyl-dopamine (NADA) (Bisogno et al., 2000; O’Sullivan et al., 2004). CB1R activation mainly drives signaling through G\textsubscript{i/o} proteins, reducing cAMP levels. Interestingly, blockade of G\textsubscript{i/o} family members by PTX revealed a low affinity coupling for G\textsubscript{s}, which results in increased cAMP levels in this specific context (Bonhaus et al., 1998; Calandra et al., 1999; Glass and Felder, 1997). Moreover, coupling of CB1R to G\textsubscript{q11} family members has also been reported upon treatment with WIN55212-2 (Lauckner et al., 2005); while coupling to G\textsubscript{12/13} subfamily has been suggested to play a role in hippocampal growth cone retraction (Roland et al., 2014). Finally, CB1R also recruits β-arrestins (Ibsen et al., 2019; Laprairie et al., 2014), which are responsible for desensitization and internalization of GPCRs, probably playing a role in tolerance to Cannabis exposure (Breivogel and Vaghela, 2015; Nguyen et al., 2012). All things considered, the range of physiological responses and signaling pathways modulated by cannabinoid receptors in various systems indicates the cannabinoid system as a viable pharmacological target.

The existence of a number of naturally occurring substances that target cannabinoid receptors allowed their use as tools to first determine the identity of their receptors and later to isolate endogenous agonists acting via these receptors. Furthermore, AEA and 2-AG discovery was characterized by the use of tissue extracts from unrelated organs: AEA from porcine brain and 2-AG from canine intestine; indicating how the choice of a tissue/organ as source of endogenous ligands is critical in the whole deorphanization process. At the beginning of the search for cannabinoid receptors, the hydrophobic nature of cannabinoids and phytocannabinoids suggested that they could act non-specifically by disrupting cellular membranes and not by activating membrane receptors. Pharmacologist should always be aware of these issues and consider possible alteration of membrane properties when studying the effect of lipid-based compounds at high concentrations.

GPR15

GPR15 was originally cloned in 1996 using homology cloning techniques, starting from the sequence of the previously identified receptors GPR7 and GPR8. Further analysis revealed high structural homology to
orphan receptor GPR25, angiotensin receptor, and APLNR (Heiber et al., 1996). GPR15 was identified as a class A orphan GPCR with a primarily role in controlling immune responses. In fact, GPR15 expression was found to be elevated in lymphocyte T cells in the spleen, colon, and lymphoid tissue, however, in human, it seems to be expressed both in regulatory and effector T cells (Fischer et al., 2016; Kim et al., 2013), while in mice it is mostly expressed in regulatory T cells (Nguyen et al., 2015). Further studies on the pathophysiological role of GPR15 were published before the identification of its endogenous ligand. For example, it was established that post-translational sulfation of tyrosine residues within the extracellular N-terminus of GPR15 enabled the binding of HIV/SIV to host cells, a mechanism analogous to what observed for chemokine receptors (Farzan et al., 1997; Farzan et al., 1999), a process that was later confirmed to have a role in the binding of the endogenous ligand (Okamoto and Shikano, 2021).

20 years after the discovery of the receptor GPR15, two independent groups finally identified its endogenous ligand in 2017 as a chemokine-like protein: C10orf99, later renamed GPR15L (Ocon et al., 2017; Suply et al., 2017). To deorphanize GPR15, Suply and colleagues co-expressed GPR15 with the promiscuous Gα16 in CHO cells. This approach is frequently applied to address the problem of studying an orphan GPCR without any previous knowledge of its G protein coupling profile. Gα16, a member of the Gq protein family, mediates an increase in cytoplasmic calcium. However, studies have shown that many GPCRs primarily coupled to other G protein families can also activate Gα16, resulting in a readily measurable mobilization of intracellular calcium. Applying this strategy, it was observed that treatments with an aqueous extract of porcine colon on GPR15-expressing cells induced receptor activation measured as a transient increase in intracellular calcium levels. Pretreatments of the aqueous extract with protease-K resulted in the loss of GPR15 activation, indicating that the ligand was most likely a peptide. Later, they fractionated the extract and verified which fraction was responsible for GPR15 activation and used MALDI-MS and Edman degradation to read the protein sequence. This analysis pointed to the product of the C10orf99 gene. C10orf99 was previously proposed as a secreted protein with potential anti-microbial activity (Yang et al., 2015) and with antitumoral properties (Pan et al., 2014). In a parallel study, Ocon and colleagues hypothesized that GPR15 could be activated by a chemotactic ligand expressed in the colon. In fact, GPR15 guides T cell homing to the colon and the developing epidermis and its sequence is related to that of chemokine receptors (Ocon et al., 2017). To test this hypothesis, they applied several gut- and skin-expressed chemoattractants and cytokines to cells expressing GPR15 without observing any binding. Then, they tested the colon-expressed polypeptide C10orf99 because a patent reported it could trigger calcium
signaling in cells transfected with GPR15. Binding and competition assays, followed by β-arrestin recruitment analysis and chemotaxis assays confirmed the role of C10orf99/GPR15L as the endogenous ligand for GPR15. Soon later, Foster and colleagues confirmed this receptor-ligand pairing and identified novel variants of GPR15L (45 and 57 amino acids) showing higher potency (Foster et al., 2019). In detail, by combining comparative genomics from over 300 species with a sophisticated bioinformatics analysis of class A GPCR sequences and structures, they discovered shared traits pointing at novel peptidergic signaling pathways. This approach led to the generation of a large library of putative endogenous peptides that was screened against a library of 21 putative peptidergic orphan GPCRs applying multiple screening assays including DMR, real-time receptor internalization with SNAP-tag, and β-arrestin recruitment using PRESTO-Tango assay (Foster et al., 2019). Further analysis of GPR15 from the same group delved into its signaling properties and defined its G protein coupling profile (Deng et al., 2022; Deng et al., 2023).

Assays based on the promiscuity of Gα15/16 (mouse/human orthologs, respectively) have become very common tools to detect GPCR activation in response to ligand exposure (Offermanns and Simon, 1995; Zhu et al., 2008). The ability of receptors to couple with Gα15/16 represents the cornerstone for the reliability of this versatile assay. However, recent studies challenged the universality of such an approach by showing that many GPCRs do not couple well with the promiscuous G15/16 (Hauser et al., 2022). This suggests that the Gα15/16 assay can potentially lead to false negative results (Hauser et al., 2022). Noteworthy, it has recently been proposed that some GPCRs may not couple at all with Gα proteins, especially current orphan GPCRs (Jang et al., 2023). This hypothesis is favored by the empirical lack of response from most of the orphan GPCRs in currently available assays, suggesting different non-canonical signaling could be activated downstream of this subset of receptors (Jang et al., 2023). Therefore, in the deorphanization process, it is important to consider both Gα protein-dependent and -independent assays, such as DMR, SNAP-Tag, PRESTO-Tango assays. Similarly, not every GPCR has been reported to couple to β-arrestin, indicating that assays based only on this readout will unlikely detect orphan receptor activation.

Conclusions

The significance of studying GPCRs stems from their involvement in mediating a wide array of physiological responses, making them prime targets for pharmacological intervention. Despite substantial progress in GPCR research, a notable proportion, roughly a fourth of the non-odorant GPCRs, remain classified as
orphan receptors (Harding et al., 2024). The lack of known endogenous ligands hinders a comprehensive understanding of their physiological roles and therapeutic potential. In this review, we provided meaningful examples of how the GPCR deorphanization process has historically followed diverse pathways, often marked by serendipitous discoveries, development of innovative methodologies, and interdisciplinary collaborations. With the described case studies, we aimed at covering the three principal scenarios encountered in deorphanization endeavors, each presenting unique challenges and opportunities. First, situations where a natural ligand, an “orphan ligand”, was identified and its biological effects uncovered but the identity of the targeted receptor was not originally known. Research efforts in these cases were sometimes hampered by the existence of multiple targets including GPCRs and ion channels requiring a preliminary dissection of their unique functional effects. Second, scenarios where synthetic ligands served as invaluable tools in elucidating receptor function and structure, paving the way for rational drug design and discovery of endogenous ligands. These case studies underscore the synergistic relationship between ligand design and pharmacological profiling in elucidating GPCR function and guiding drug discovery efforts. Lastly, instances where the identity of a natural ligand remained elusive, despite evident biological effects associated with the expression of an orphan receptor. This scenario underscores the intricate interplay between biochemical assays, behavioral studies, and computational approaches in narrowing down potential ligand candidates and deciphering their interactions with the receptor of interest. Another level of complexity is represented by orphan receptors whose ligands are constantly present in the culture media, and as such may result in desensitization and lack of response in functional assays. The history of GPCRs and the process of their deorphanization is rich in serendipitous discoveries or unexpected cross-reactivity leading to unveiling novel ligands, highlighting the importance of leveraging diverse screening approaches to uncover hidden pharmacological profiles. By integrating historical perspectives with contemporary advancements, we offered a comprehensive roadmap for unraveling the complex landscape of GPCR-ligand interactions and harnessing their therapeutic potential.
Authorship Contributions
Wrote or contributed to writing of the manuscript: Franchini L., Orlandi C.

Footnotes
This study was supported by the Drug Targets and Mechanisms Program of Excellence from the Department of Pharmacology and Physiology, University of Rochester (to C.O.); University Research Award, University of Rochester (to C.O).

No author has an actual or perceived conflict of interest with the contents of this article.
This article contains no datasets generated or analyzed during the current study.

Figure Legends:
Figure 1: Scheme of alternative GPCR deorphanization scenarios. Created with BioRender.
REFERENCES


Table 1. Milestones in the pairing of representative ligands with cognate GPCRs.

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