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Orphan G-protein Coupled Receptors

Can we deorphanize the remaining orphans despite all the challenges?

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1 Abstract

G-protein coupled receptors (GPCRs) play a key role in a broad range of biological processes by binding to a wide variety of signaling molecules, which have resulted in 34% of all FDA-approved drugs which target GPCRs. The human genome encodes for approximately 800 GPCR members of which about 140 non-olfactory receptors remain orphans with an unknown function and endogenous ligand. Despite prolonged efforts to deorphanize the unresolved receptors, they remain orphans until this day. By studying scientific publications, this thesis has clarified the challenges with the deorphanization of GPCRs to explain why there are still so many orphan GPCRs when they have confirmed involvement in so many human disorders.

Keywords: Deorphanization, Heterodimerization, High-throughput screening, Orphan G-protein Coupled receptor, Pseudogenes, Reversed pharmacology

2 Introduction

G-protein-coupled-receptors (GPCR) are an enormous protein family expressed in all body tissues, with various physiological functions (Zhang, Zhao and Wu 2015). Molecular signaling by the receptors is crucial and has evolved into a complex network of cell-to-cell interactions, activated by a comprehensive array of natural ligands, regulating all aspects of human physiology (Civelli, o.a. 2006). All the receptors included in the GPCR family have their unique characteristics, even though they all share a standard structure. There are two criteria to ensure that a protein can be classified as a GPCR. The first requirement includes seven α-helices located across the plasma membrane, resulting in the seven transmembrane domains (7TM), which create a binding site for ligands on the extracellular site. The second requirement covers the ability of the receptor to bind to a separate G-protein, causing an interaction with several other proteins inside the cell – termed second messengers (Fredriksson, Lagerström and Lundin, et al. 2003).

The human genome encodes for approximately 800 GPCR members, of which ~ 400 are odorant. Most of the 400 non-odorant GPCRs have an identified ligand, while over 100 GPCRs are orphans with unidentified ligands and unclarified functions despite extensive research efforts into their potential functions expensive and prolonged efforts (Ahmad, Wojciech and Jockers 2015). Deorphanization refers to the process where the ligand and the function are

detected. GPCRs have a strong association with human pathophysiology and have therefore been intensively studied to deorphanize the receptors to develop drugs that target the receptors to improve medical care (Hauser, et al. 2017).

The ligands are commonly present in the extracellular environment or released by another cell and thereby work as the first messengers in cell signaling by GPCR. A particular ligand is directly dependent on the structure, function, and location of the receptor, and it can thereby vary from small molecules like ions, and amino acids to larger like biogenic amines, lipids, nucleotides, peptides, proteins, and olfactory molecules (Ahmad, Wojciech and Jockers 2015).

2.1 Classification

Two central classification systems are used to classify GPCR into different subfamilies: the GRAFS and the A-F systems. Fredriksson et al. (2003) established the GRAFS system when they analyzed 802 unique GPCRs encoded by the human genome to construct a phylogenetic tree to design a classification system adapted for humans. Their data collection and schematic presentation of all GPCRs in the human genome resulted in five main families: glutamate, rhodopsin, adhesion, frizzled/taste, and secretin: GRAFS. The other classification system includes all GPCRs proven to bind G-proteins, in which there are two subfamilies not found in humans: classes D and E (Fredriksson, Lagerström and Lundin, et al. 2003). This thesis will henceforth refer to the GRAFS system.

2.1.1 The Glutamate Receptor Family

This subfamily consists of 22 unique receptors corresponding to class C in the A-F classification system. The family of glutamate includes two γ aminobutyric acid receptors, eight metabotropic glutamate receptors, five taste receptors, and seven orphan GPCRs. An attribute of the family is the relatively long N-terminal, which also provides a domain for ligand binding. A surprising exception for metabotropic glutamate receptors is allosteric ligands that bind to some of the transmembrane domains (TM3, TM5, TM6, TM7) (Lagerström and Schiöth 2008).

2.1.2 The Rhodopsin Receptor Family

The rhodopsin receptor family corresponds to group A in the A-F classification system, and it constitutes the most studied and the largest GPCR family with 670 receptor proteins in humans. In contrast to the other families of GPCRs, the rhodopsin has by far the shortest N-terminus. This is relevant for the ligand binding, which instead is located between the transmembrane domains for most of the rhodopsin receptors. Regarding the ligands for the

receptors, studies show a huge variety of ligands and a highly heterogeneous receptor structure due to the size of the family. All variants can therefore be divided into four separate groups based on the human genome – α, β, γ, and δ (Lagerström and Schiöth 2008).

There are, however, many similar qualifications between the groups. The endogenous ligand that binds to the receptors is quite the same in several groups. All four subgroups are found to be peptide-binding receptors; three of the groups can bind lipid-like compounds. Some differences are yet worth mentioning; the α group consists of 89 receptors, including amine binding GPCRs, peptide binding and prostaglandin receptors. The β-group includes 35 members, mainly peptide-binding receptors, while the γ group includes bindings for both peptides and lipid-like compounds. The last group, δ, is the large group of ~ 460 olfactory receptors and 58 non-olfactory receptors like purin receptors and glycoprotein receptors (Fredriksson, Lagerström and Lundin, et al. 2003).

2.1.3 The Adhesion Receptor Family

The second-largest GPCR family encoded by humans consists of 33 members, whereby many have sequence similarities with the Secretin Receptors Family (described below). (Lagerström and Schiöth 2008). The name for the family is related to their extremely long N terminal. However, the length varies widely, from 200 to 2800 amino acids long. The N termini are very likely to interact and attach to cells nearby via adhesion (Fredriksson, Lagerström and Lundin, et al. 2003). The ability for adhesion is due to the high concentration of Ser and Thr residues that form a rigid structure erecting from the membrane, based on O- and N-glycosylation sites. The GPCR proteolytic domain in their N-terminus is unique to the Adhesion receptors, even though no clear function has been found for the GPS (Fredriksson, Lagerström and Höglund, et al. 2002).

2.1.4 The Frizzled/Taste2 Receptor Family

This group of receptors is created by two clusters of receptors - 11 frizzled receptors and 33 taste receptors - in the human genome (Schiöth and Fredriksson 2005). As the clusters do not share any distinct similarities, it is questionable if they even share an evolutionary history. However, the branches show a high bootstrap value in the phylogenetic tree. One reasonable cause is some consensus sequence between the clusters, which is not shown in any other subfamily of GPCR (Fredriksson, Lagerström and Lundin, et al. 2003).

The frizzled receptor has a relatively short N-termini with approximately 200 amino acids with preserved cysteines that likely contribute to the curled and twisted Wnt ligand, which is

the basis for the term frizzled. The frizzled receptors' function is to control the cell fate, distribution, and development of functional complexity during metazoan development (Schiöth and Fredriksson 2005). In contrast, the taste receptors are located at the tongue and palate epithelium and function as bitter taste receptors (Chandrashekar, et al. 2000).

2.1.5 The Secretin Receptor Family

The smallest family of GPCRs is the secretin receptor family; all the 15 members have their binding site at the extracellular domain, which in turn only binds peptide hormones. For this reason, the secretin receptors represent significant potential for drug development due to their homeostatic role. Thus, the minimalist size of their peptide ligands challenges the development of drugs targeting these receptors (Lagerström and Schiöth 2008).

2.2 Impact of GPCRs on drug development

Approximately 480 drugs on the market target 107 unique GPCRs, which constitutes 34% of all drugs approved by the US Food and Drug Administration (FDA). New drug candidates are continually in clinical trials, and the number of approved drugs for GPCR is growing, which indicates that the receptors are an ideal drug target. The interest for GPCR-targeted candidates shifted over time, with schizophrenia, depression, and hypertension previously in focus. Drugs that target GPCRs involved in disorders such as Alzheimer's disease, obesity, multiple sclerosis (MS), and diabetes type 2 have been highlighted in recent years. Central nervous system disorders remain highly represented in drugs targeting GPCRs, due to non-functional GPCR expressed in the cerebral cortex, often resulting in several psychiatric disorders (Hauser, et al. 2017).

Currently, only ~100 of all GPCRs encoded by humans have a current target drug approved and used in clinical practice. Therapeutic drugs can affect the receptors as an agonist or antagonists, so 400 non-odorant GPCRs in humans represent roughly 800 potential therapeutic medicines. Less than 13% of the drugs that involve human GPCRs have successfully been identified (Congreve, de Graaf, et al. 2020).

2.2.1 Deorphanization and aim of this thesis

Several factors influence the drug discovery for GPCRs, whereas the deorphanization of orphan GPCRs constitutes one of the future opportunities since it yields new targets. The general goal of this thesis is to present the significance of the deorphanization of orphan GPCRs and how they may contribute to further drug development. Moreover, the report will present

the development of the deorphanization of orphan GPCRs over time to identify the deficiencies and significance of the methods and how it implicates therapeutic drugs.

Questions at issue

- Why are there still so many orphan GPCRs when they have confirmed involvement in so many human disorders?
- Can all remaining orphan GPCRs be deorphanized and included in drug development?

2.2.2 Limitations

The search for novel GPCRs is inevitably the first step for later deorphanization and drug development associated with GPCRs. Yet, designed experiments for detecting new receptors will only be briefly mentioned. This literature report aims to examine experiments designed for deorphanizations of identified GPCRs.

Regarding the olfactory GPCRs (~ 400), a limited number of studies have been conducted to deorphanize the receptors. Some orphan olfactory receptors have been paired with its ligand, but the majority will remain orphans due to several factors. The modest interest in studying these orphan olfactory GPCRs is based on the limited association with human pathology and disorders. Further, these receptors have a high rate of development due to a high variation across mammalian species, which challenges deorphanization. Therefore, the potential for deorphanize the orphan olfactory GPCRs is restricted (Lagerström and Schiöth 2008) and will be excluded from this report.

3 Method

This thesis was a methodical and critical study of publications in scientific journals. The choice of articles was based on their title and abstract to ensure that they were relevant to the literature report, and a quality review of the obtained articles was implemented to achieve a critical literature study.

3.1 Data collection

Information was provided from several different databases such as PubMed, Web of Science, Science Direct and Google Scholar. At the start of the project, the focus was on collecting literature reviews to get an overview of the topic. Search terms were used in the initial searches, occasionally combined with subject headings, to achieve more relevant results. The

later articles were primary research articles to achieve narrow and specific information. Data articles, discussions, and product reviews were used from the databases to supplement the report.

3.1.1 Subject heading

A subject heading was prioritized to achieve precise and narrow results in the literature research and ensure that synonyms and spelling variations were included. Subheadings were used if offered by the database to increase the focus on the subject.

Search terms

"Receptors, G-Protein-Coupled/classification" OR "Receptors, G-Protein-Coupled/drug effects" OR "Receptors, G-Protein-Coupled/history" OR "Receptors, G-Protein-Coupled/pharmacology" OR "Receptors, G-Protein-Coupled/physiology" OR "Receptors, G-Protein-Coupled/physiopathology" OR "Receptors, G-Protein-Coupled/therapeutic use"

3.1.2 Free-text searching

Free-text searching was constructed in the early stages of the thesis or if subject headings were not possible to use. The search needed to consider synonyms, different spellings, and grammar variations to reduce the risk of excluding relevant results.

Search terms

GPCR, G-protein-coupled receptors, 7TM, seven-transmembrane receptor, orphan, orphan receptor, deorphanized, endogenous ligand, ligand, reverse pharmacology, high-throughput screening, heterodimerization.

4 Main Results

NC-IUPHAR[1] has established some standards to define a GPCR as deorphanized. Introducing the first point; to consider a proper endogenous ligand for a receptor, reproducibility needs to be demonstrated. This is achieved when at least two independent research groups demonstrate interaction and activity between the ligand and the receptor. Furthermore, the result needs to be consistent with the physiologic function. Even if it is achieved, if other research groups fail to reproduce the pairing, NC-IUPHAR can retract the reported results (IUPHAR/BPS Guide to PHARMACOLOGY n.d.).

Furthermore, the ligand needs to be specific to a receptor, which implies binding with high affinity and in a saturable manner and displaceable kinetics. The ligand-binding also needs to be able to demonstrate a physiological response. Functional assays should fulfill these criteria both *in vitro* and *in vivo*. Further evidence must demonstrate that the ligand is present in the tissues with an appropriate concentration (Davenport, et al. 2013).

4.1 Deorphanization of GPCRs – trends and strategies

The first structure of a G-protein coupled receptor was determined in 1983 by isolation and cloning of cDNA for further analysis of the nucleotide sequence, which resulted in a complete amino acid sequence for the G-protein coupled receptor rhodopsin (Nathans and Hogness 1983). The peptide sequence was confirmed the same year by (Hargrave, et al. 1983), who also could determine the protein's structure. A significant breakthrough for GPCRs came when Dixon et al. constructed a genomic library for screening which led to an unexpected finding; both β2-adrenoceptor and rhodopsin shared a sequence resulting in the seven-transmembrane structure. The concept of a GPCR family was established in parallel with the knowledge that homology screening techniques could identify further novel GPCRs (Dixon, et al. 1986). A couple of novel techniques were developed in the early 1990s; PCR-based homology screening and reversed pharmacology, resulting in a revolutionary GPCR deorphanization (Civelli, o.a. 2006).

[1] Nomenclature and Standards Committee of the International Union of Basic and Clinical Pharmacology

Reversed pharmacology turned out to be a successful strategy in terms of the deorphanization of GPCRs. The method uses cDNA from the orphan GPCR of interest and introduces this into the nucleus by penetration with DNA-coated particles – referred to as exogenous expression. Various ligands are used for interaction with the receptors to later observe potential activation by a response in the intracellular second messengers. Some adaptations from reversed pharmacology resulted in the variant called `the orphan receptor strategy´; orphan GPCRs were repeatedly exposed to tissue extract to measure changes in second messenger responses and obtain an isolated ligand (Levoye och Jockers 2008).

The parallel work of finding new GPCRs and pairing orphan GPCRs with their ligand and function proceeded at a high rate until the 2000s. The number of orphan GPCRs increased steadily and dominated the known potential ligands. This led to the conclusion that the orphan GPCRs should function with ligands that have not yet been characterized, and the process of identifying novel ligands began. At the beginning of the era, the search for novel ligands required countless repetitive assays, supported by the pharmaceutical industry. The first search started in tissue extract to identify novel ligands to later develop in studying complex molecular mixtures, using the orphan receptor as a bait for the ligands (Civelli, o.a. 2006).

One of the first discovered novel natural ligands was nociception/orphaning FQ, paired with the orphan GPCR ORL-1. The ligand was found because of the high sequence similarity (65%) between the receptor ORL-1 and some opioid receptors (Civelli, et al. 1997). If the sequence identity between different receptors reaches ∼45% or more, they are likely to share a common ligand (Marchese, et al. 1999). Opioid receptors interact with peptidergic ligands. The high sequence similarity between the receptors leads to the assumption that the (previous) orphan ORL-1 also binds to a peptidergic ligand and shares a similar physiological function. Researchers isolated natural ligands from the hypothalamus to study if ORL-1 interacts with a peptidergic ligand, where the receptor is expressed. The material was processed and analyzed by mass spectrometry and sequenced by Edman degradation to confirm the primary structure of the ligand. The sequence screening revealed that the interacting ligand to ORL-1 was a peptide, just as for opioid receptors. Civelli et al. could not monitor any activity between the newly found peptide and opioid receptors simultaneously as standard ligands for opioid receptors could not bind to the ORL-1 receptors – revealing a significant difference between the receptors (Civelli, et al. 1997). Similar studies have been implemented to identify ligands in the hypothalamus. Orexin-A and Orexin-B are two examples of peptides that regulate feeding

behavior and energy homeostasis, expressed within and around the lateral and posterior hypothalamus (Sakurai, et al. 1998).

Later, several laboratories started approaching screening libraries to explore unexpected or novel ligands for the receptors. The process of screening libraries required the development of high-throughput assays, a successful process that eventually was used to randomly screen orphan GPCRs against libraries containing ligands that were not paired to any receptor. The years of randomly screening orphan GPCRs against large libraries resulted in an "industrial" era of deorphanization where 40 GPCRs became deorphanized (Civelli, o.a. 2006).

The number of deorphanized GPCRs has varied over time, with the most rapid era of deorphanization occurred between 1990-2005 (Civelli, o.a. 2006), (Oh, et al. 2006), (Laschet, Dupuis and Hanson 2018). Since then, published deorphanizations of GPCRs have steadily decreased to just a few findings per year. Laschet et al. claim in their review in 2018 that the decrease in deorphanization can be explained by reasonable causes rather than as a period of crisis. They declare the field to be relatively saturated after many years of intense studies and discoveries. To proceed deorphanization of GPCRs, new technologies and perspectives are required to resolve the remaining non-odorant orphan GPCRs (Laschet, Dupuis and Hanson 2018).

4.2 The Potential of Orphan GPCR

Even though GPCRs are usually highlighted as the most potential source of drug targets, roughly 120 GPCRs are still orphans. Fortunately, these orphan receptors' biological role and pathophysiology have been studied through several studies such as siRNA screening (Ku, et al. 2012) and gene knockout in mice to understand their function and involvement in disorders. Several GPCRs have been identified to regulate activities associated with diseases or be directly involved in pathophysiological conditions (Ahmad, Wojciech and Jockers 2015).

The major highlighted disorder linked to orphan GPCRs is different forms of cancer. Triple-negative breast cancer (TNBC) is associated with a harmful medical state in 20% of all breast cancer cases in women. TNBC has a high genetic heterogeneity, which results in a lack of an operating therapy. However, Feigin et al. identified the receptor GPR161 as a prognostic biomarker for TNBC due to its significant overexpression in breast tissue in patients with TNBC. The mutations of GPR161 modified the ligand-binding site for the receptor and the interaction site with β-arrestins, which are highly relevant for the development of TNBC and compose a potential drug target (Feigin, et al. 2014).

GPCRs have an abundant expression in the brain, resulting in several promising targets for neurophysiology and neurogenerative disorders such as Parkinson's disease and Schizophrenia (Ahmad, Wojciech and Jockers 2015). The orphan G-protein coupled receptor 6 (GPR6) is likely to be involved in Parkinson's disease due to its association with cyclic adenosine-3´,5´-monophosphate (cAMP) formation in striatopallidal neurons. Ockl et al. investigated in 2014 the effect of decreased expression of GPR6 in mice, resulting in a behavioral phenotype with increased muscle activity in combination with a reduced abnormal involuntary movement, typical symptoms for a patient with Parkinson's disease. The GPR6 could therefore be a promising target for the treatment of Parkinson's disease (Oeckl, Hengerer and Ferger 2014). A more significant number of orphan GPCRs have been demonstrated to modulate multiple mechanisms involved in striatal-based disorders. The orphan GPCR GPR88 demonstrates one example due to its high expression in the striatum. GPR88 has been proven to modulate the striatal dopaminergic system in vivo and in vitro assays in mice (Logue, et al. 2009). Schizophrenia is, in turn, associated with abnormal sensitivity against dopamine (Logue, et al. 2009). The modulatory role of GPR88 can hence act as a possible target for pharmacological treatment of schizophrenia (Logue, et al. 2009).

Orphan GPCRs play a central role in energy metabolism, including modulation of diet-induced obesity and insulin sensitivity. Mice showing homozygous negativity for GPR82 have reduced weight, food intake and increased insulin sensitivity and glucose tolerance. Accordingly, a functional GPR82 is probably required for optimal energy homeostasis and fat deposition. The studies suggest that GPR82 can act as a potential target for diabetes and obesity by regulating an antagonist to reduce food intake. However, the authors of the discovery underline the complex mechanisms of energy metabolism and that GPR82 is not the only factor that affects it – further studies are needed (Engel, et al. 2011). Additional orphan GPCRs are proven to influence energy metabolism by regulating several factors, e.g., GPR26, GPR21, GPR27 and GPRC5B. These receptors can emerge as future drug targets for diabetes and other metabolic disorders (Ahmad, Wojciech and Jockers 2015).

4.3 Challenges to deorphanize orphan GPCRs

Some studies of orphan GPCRs suggest a more complex perspective; receptors can function beyond a ligand-dependency – suggesting a need for complementary assays that include physiological responses in the absence of ligands.

4.3.1 Heterodimerization

Several GPCRs can modulate the function of other receptors through heterodimerization. The interaction between two separate GPCRs to form a functional and active combination is essential for some receptors. For the $GABA_B$ receptors, heterodimerization is essential. Coexpression between $GABA_BR1$ and $GABA_BR2$ results in the formation of the functional receptor $GABA_B$; when expressed alone, it will not reach the cell surface and will not bind to its ligand. Similar examples for $GABA_B$ are found in other receptors. In the adrenergic receptor family, $\alpha_{1D}AR$ does not display any physical function when expressed alone. Instead, it forms heterodimerization with $\alpha_{1B}AR$ and β_2AR, resulting in a functional activity (Prinster, Hague and Hall 2005).

Functional complexes of orphan GPCRs are also relevant between orphan GPCRs and other cellular proteins. Proteomic techniques are traditionally associated with studies of proteins, whereas affinity-based procedures can identify the interaction between two different proteins. GPR50 is an orphan GPCR involving mental disorders and lipid metabolism, located in the pituitary, hypothalamus, and hippocampus in the mammalian brain. Although GPR50 has no identified ligand, other interaction-dependent functions have been determined. Firstly, GPR50 can form interactions with the MT1 and MT2 melatonin receptors, where interaction with MT1 prevents melatonin binding and signaling. Secondly, GPR50 has been suggested to interact with the potent neuronal inhibitor NOGO-A, causing a significant difference in neurite length and affecting the neurite outgrowth (Grünewald, et al. 2009).

The evidence for interactions between GPCRs opens the possibility that some GPCRs lack an endogenous ligand. This theory challenges the deorphanization of all orphan GPCRs; simultaneously, it is difficult to prove that some receptors lack an endogenous ligand (Lagerström and Schiöth 2008).

4.3.2 Reproducing a state of interaction

One criterion for the deorphanization of GPCRs is the ability to reproduce and confirm the results by other research groups. Owing to the criteria, ligands for some orphan GPCR have been highly discussed since some groups are unable to confirm a suggested ligand. The receptor GPR3 constitutes one example of a challenged deorphanization, sphingosine 1-phosphate (S1P) has been suggested as a ligand for the receptor GPR3 to treat Alzheimer's disease. Several groups are still incapable to verifying the results, and GPR3 consequently remains an orphan receptor. Resembling cases are found in the orphan GPR6 and GPR12 – suggested targets for

Alzheimer's disease, Parkinson's disease, cancer metastasis, and infertility (Davenport, et al. 2013).

Similar examples exist in other subfamilies of GPCRs, for which only one single publication could demonstrate a ligand to the receptors with unsuccessful attempts to reproduce the findings by an independent laboratory. GPR3, GPR6 and GPR12 belong to the Rhodopsin subfamily, where there is a total of 35 orphan receptors missing replicas on the discoveries. In the Adhesion subfamily, seven GPCRs remains orphan due to a lack of confirming publications. Only one receptor remains orphan in the Glutamate subfamily, GPRC6A, which is reported to respond to basic amino acids (IUPHAR/BPS Guide to PHARMACOLOGY n.d.).

4.3.3 Novel perspectives on GPCRs for drug discovery

As mentioned previously, ~100 GPCRs are targeted by approved drugs on the market; of these, 46 GPCRs have a determined protein structure. X-ray crystallography has been the most successful strategy to determine GPCR structure in the latest two decennia, which generates an understanding of the interaction between the ligand and the receptors, thus resulting in increased knowledge about the molecular pharmacology of the receptors. Thus, X-ray can only visualize very fixed structures and lack the capacity to determine a structure of a more dynamic structure, as GPCRs have. Thereby, X-ray crystallography has facilitated the development of other techniques, such as electron cryo-microscopy, which likely will outcompete X-ray crystallography regardless of its success. In contrast to X-ray crystallography, electron cryo-microscopy uses direct electron detectors and modern computer programs to achieve a resolution of 3.0-3.5 Å – adapted to determined structures of smaller receptors. In contrast to X-ray crystallography, it also has the ability to visualize dynamic structures of proteins (Congreve, de Graaf, et al. 2020).

Even if the electron cryo-microscopy can determine the structure of the protein with a better resolution, the process could take several weeks, which is in contrast to a couple of hours when using X-ray crystallography for structure determination. Except for the different time scales for structure determination between the two methods, electron cryo-microscopy remains the one that is the best suited for later structure-based drug development (SBDD). Partly because of its higher tendency to determine the structure of smaller, dynamic proteins and its potential for improvement in the near future (Congreve, de Graaf, et al. 2020).

There could be a potential for drug discovery based on the determined structure of the receptor, referred to as structure-based drug design (SBDD). The method aims to purify the

receptors from the cell membranes to determine their structure and exhibit a detailed image of the binding site for ligands. Understanding the interactions leads to opportunities to reveal ligands that fit the receptor, facilitating the precise design of a drug targeting the receptor (Congreve, de Graaf, et al. 2020).

Despite the theoretical potential of SBDD for GPCRs, only three examples of ligands have been identified by using the method. Congreve et al. identified 1,2,4-Triazine as a potent ligand to adenosine A_{2A} receptor for possible treatment of Parkinson´s disease (Congreve, Andrews, et al. 2012). Secondly, the biopharmaceutical company Sosei Heptares discovered multiple agonists for the M_1- and the M_4 receptor in 2016 (Congreve, de Graaf, Swain, & Tate, 2020). The same company is in charge of the third example, discovering the negative allosteric modulator HTL0014242 targeting the orphan receptor mGlu5 (Christopher, et al. 2015). Even though the few examples of discoveries by using SBDD, it is a new approach that has the potential to provide new successful discoveries in the near future.

Another novel perspective for drug discovery includes the evolutionary history of the receptors. Obtained mutations during the evolution of GPCRs have proven to be relevant as pseudogenes – DNA sequences with acquired mutations that leave the receptors non-functional. The phenomenon is thought to occur across gene duplications where the new copy accumulates the mutation over generations. In this way, a GPCR gene can be non-functional in some species while functional in others. Moreover, the same principle can be applied to different human populations – also known for a polymorphic mutation. The orphan receptor GPR33 is one of the identified pseudogenes in humans, a receptor that occurred in mammals 125-190 million years ago. Even though mutations resulted in a nonfunctional receptor in humans 1 million years ago, analysis shows that the allele of GPR33 still is expressed in the human population. Interestingly, the GPR33 allele remains intact in a small fraction of the human population – present all over the world and confirming an inactivation before the last migration out of Africa (Römpler, et al. 2005).

A few more receptors have been proposed to be pseudogenes in humans; GPR42, GPR79, GnRH2, TAAR2 and TAAR9. All of them contain mutations that have resulted in a nonfunctional receptor in one or several species. The extent and their functional role vary, yet they can be considered important for further studies (Davenport, et al. 2013).

5 Discussion

This thesis has clarified some of the challenges associated with the deorphanization of GPCRs, although some challenges are not mentioned. During the work of the thesis, the reason as to why there remains so many orphans are to some extent answered. Taken together, these perspectives will give some answers to the questions at stake for the thesis.

Even though all cells express a GPCR, the level of expression may differ markedly between cells and individuals. If a cell expresses deficient levels of a particular GPCR, it may challenge the ability to measure activity after an interaction with a ligand. The method referred to as reversed pharmacology has been used well and has resulted in a high quantity of deorphanized GPCRs; despite this, the method has difficulty measuring minimal responses. Therefore, it may be relevant with a resolution that stimulates the expression of the specific GPCR to easily detectable levels as a future step towards more deorphanization of GPCRs.

Nor is it given that a GPCR is expressed and located only on the cell membrane. Some GPCRs have been found in the cell nucleus the endoplasmic reticulum (ER) or the Golgi apparatus, whereupon a compound is needed for transporting the receptor to the membrane, where it integrates with the ligand and performs its function. For example, the receptor $GABA_BR1$ will remain non-functional and located at the ER in absence of $GABA_BR2$ – their heterodimerization is essential since it results in the transportation of $GABA_BR1$ to the cell surface (Prinster, Hague and Hall 2005). The phenomenon results in having an additional step that needs to be investigated before the given ligand can be identified for a successful deorphanization of the receptor.

Another approach worth exploring is whether there are alternative intracellular ligands to the receptor, which activate a process that needs to be measured extracellularly. At present, the analyses are adapted to show how the ligand integrates with the receptor extracellularly, and the response is measured intracellularly. Based on the different subfamilies of GPCRs, knowledge has been gained about where on the receptor the ligand tends to bind. Through further structure-based investigations, it might be rewarding with more details about the position and the approach regarding the interaction, in order to find new alternative interactions.

A high potential may exist in structure-based drug development (SBDD), even though the method so far only has resulted in three identified ligands. One future possibility for the non-functional GPCRs due to pseudogenes and the ligand-independent receptors would be

worth exploring whether their structure can be determined in detail to develop synthetic, surrogate ligands later.

Apart from the ligand-independent receptors, some studies suggest a few receptors encoded in our cells, but whose function and ligand have been selected for during evolution - pseudogenes. The phenomenon of pseudogenes is demonstrated to be relevant through the evolution of GPCR, leaving nonfunctional GPCRs in some species while they may remain functional in others. In order to later succeed in finding a ligand, studies need to expand to include additional species, even if they may be unexpected. Functionality variations have also been detected within one species but between different populations. Such as the GPR33 allele in humans, which is intact in small fractions of people scattered across the world (Davenport, et al. 2013), (Römpler, et al. 2005).

More examples of pseudogenes in GPCRs should exist, which unfortunately challenges the process of deorphanize all receptors. How many GPCRs are relevant as pseudogenes are currently unknown, but in parallel with the increased knowledge, they can, like the ligand-independent orphan GPCRs, also contribute to reducing the number of possible deorphanizations; and should therefore be omitted from the approximately 140 orphan GPCRs often mentioned in literature.

Regarding the criteria for an accurate deorphanization, they should be reviewed as for the criterium whether deorphanization needs to include an identification of an interaction between ligand and receptor that is also linked to a physiological function. Several studies support the fact that some GPCRs have a ligand-independent function. Heterodimerization is a variant, functional complexes between other proteins in a cell membrane is another, processes for the GPCR in question to be activated and functional. The evidence suggests that many GPCRs lack an endogenous ligand, which challenges deorphanization according to the criteria for deorphanization. The consequence is to either accept that the ligand-independent receptors will continue to be considered orphans; otherwise, the requirements should be modified. When discussing orphan GPCRs, olfactory GPCRs are excluded all too often, which may also be relevant for ligand-independent orphan GPCRs. If so, one may find the number of orphan GPCRs lower than what the information first suggests.

As with everything in research, it is easier to prove that something is false than prove that something is true. Therefore, it is almost impossible to prove that a receptor completely lacks a ligand. At present, no discoveries have been published about hybrid receptors, which both have

a ligand-independent function as well as another ligand-dependent function – however, such findings may occur in the future.

Deorphanization of GPCRs is a fundamental mission due to their great potential in drug discovery. The statement is partially confirmed because 34% of all approved drugs by the FDA target GPCRs. The high percentage is often used as an argument that GPCRs have significantly more potential. However, a high proportion of drugs on the market does not have to mean that there is much more to contribute. Just because GPCRs have an excellent potential for medicine does not mean that the remaining orphan GPCRs might be involved in future drug treatments. Today, several GPCRs can be used as biomarkers for different disorders such as cancer and Parkinson's disease. Identifying overexpression or underexpression can therefore be considered a major step forward to improve medical care.

Unfortunately, during the work on my thesis, no shared database has been found that records all data regarding GPCRs. Therefore, the numbers vary greatly regarding how many orphan GPCRs remain and the information concerning how the deorphanization of GPCRs has been throughout history. The number of reported GPCRs tends to be obtained from published literature, resulting in a high risk of miscalculations as the acquired articles with data are found manually and by cross-referencing. Unfortunately, it is impossible to include all the articles published around a particular GPCRs, which consistently leads to some information being lost. Fortunately, the IUPHAR/BPS Guide to Pharmacology exists - but even this database lacks a practical basis. Irregular updates and poor strategic reporting results in misleading information. The numbers presented in my thesis are taken from other research groups that have manually compiled information about the total amount of orphan GPCRs. However, it leaves much room for information to lose its basis if it is reused for several years without being critically inspected.

The lack of trustworthy information contributes to difficulties in keeping up to date on which GPCR is in an ongoing deorphanization process. According to the criteria of a deorphanized receptor, it is required that two independent research groups succeed in validating an interaction between the ligand and the receptor, which additionally is consistent with the physiological function. Table 1 features the number of receptors (in parentheses) that a research team has managed to identify a ligand for, but which no other group has managed to reproduce. They are therefore remaining as orphan GPCRs. Many discoveries occur regularly but without being validated. According to my previous paragraph, the information about which GPCRs are at which stage of deorphanization needs to be available and reliable for others to continue the process towards deorphanization.

Table 1. *The number of deorphanized GPCRs and the remaining orphan GPCRs are associated with their given subfamily. The numbers in parentheses represent orphan receptors for which a ligand has been identified by one publication. Adapted from (IUPHAR/BPS Guide to PHARMACOLOGY n.d.).*

Receptor Family	**Glutamate**	**Rhodopsin**	**Adhesion**	**Frizzled**	**Secretin**
Deorphanized receptors	12	197	-	11	15
Orphan receptors	8 (1)	87 (54)	26 (6)	-	-

Another perspective on why, after so many years, a high proportion of orphan GPCRs remains: new GPCRs are regularly identified. When the work with deorphanization of GPCRs takes place in parallel with the identification of new GPCRs registered in a merged database, it challenges the distinction and may give the impression that the number of deorphanization of GPCRs has not decreased. The rate of deorphanization may have been relatively stable over the years, but the fact that new receptors have been identified may have seemed misleading to some conclusions.

The above perspectives demonstrate that a function and ligand to an orphan GPCRs do not necessarily need to be limited to classical receptor signaling activity. Instead, it should be extended to include multiprotein complexes, pseudogenes, and other mentioned phenomena in this thesis. This is especially true since there is a belief that more discrepancies will be discovered in the near future, which is confirmed by the fact that they are steadily increasing. The focus of the future will probably be on identifying functions that are far beyond ligand-dependent functions. The new perspectives argue for modifying new assays and criteria to deorphanize the remaining orphan receptors successfully.

6 Societal Considerations

Because G-protein-coupled receptors are closely associated with medical treatments and drug development, this literature report is relevant to increasing knowledge about orphan GPCRs and possibly contributing to the further development of biotechnologists associated with deorphanization. The decrease in deorphanization can be questioned, especially since the receptors are relevant for drug development. This thesis aimed to explain the challenges of deorphanization, which has been clarified by explaining how strategies have changed

historically - which in turn leads to understanding the decrease. Knowledge of all aspects of deorphanization needs to be considered, and new perspectives should be reviewed to understand how the deorphanization of GPCRs may develop in the future. The long-term goal is to increase knowledge about the deorphanization of GPCRs to provide new drugs which target the receptors. Many diseases lack effective medical treatment due to a not fully understood mechanism, as well as the relevant drugs that would target the receptors involved. As GPCRs are involved in the majority of the body's physiological principles, it is a given that more knowledge about the receptors is relevant for further drug development.

The aim of this thesis has a close connection to the third global goal, “Good Health and Well-being – ensure healthy lives and promote well-being for all at all ages”. The third global goal includes thirteen sub-goals, whereas subgoal 3.4 would be the most relevant to this thesis. The target is to reduce the number of deaths associated with non-communicable diseases and promote mental health and well-being. This thesis has explained the connection between some of these diseases and drugs which target GPCRs.

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