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MATHEMATICAL MODELING OF OLIGOMERIZATION AND BIASED SIGNALING OF G-PROTEIN-COUPLED RECEPTORS

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Doctoral Thesis

Mathematical modeling of oligomerization and biased signaling of G-protein-coupled receptors

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CERTIFICA

Que la tesis doctoral titulada "**MATHEMATICAL MODELING OF OLIGOMERIZATION AND BIASED SIGNALING OF G-PROTEIN-COUPLED RECEPTORS**", presentada por el señor BIN ZHOU dentro del programa de doctorado de Neurociencias de la Universitat Autònoma de Barcelona, ha sido realizada bajo su dirección y, considerándola concluida, autoriza su presentación con el fin de que sea juzgada por la comisión correspondiente.

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Abstract

G-protein-coupled receptors (GPCRs) play very important roles in a great variety of biological processes. They are located in the membrane and mediate the signaling pathways in the cell. It is widely accepted that these receptors often form oligomers which may have significant physiological functions. In addition, one GPCR may act at several downstream signaling pathways, and these pathways can be differentially activated by the ligand of the receptor. The present thesis tries to provide new mathematical tools for the understanding of these phenomena. There are two new mathematical models for GPCR oligomerization and one novel framework for biased signaling in the present thesis.

Firstly, a new mathematical model is proposed for the receptor heterodimer. This heterodimer model can be employed to dissect the impact of the two ligands which are respectively bound to the two protomers in the heterodimer on the downstream signaling pathways of the heterodimer. Secondly, a new mathematical model is presented for the receptor homodimer. This homodimer model can be utilized to analyze a wide range of dose-response curves of the ligands binding to the receptor homodimer and the biased signaling which is dependent on ligand concentration. Thirdly, a novel conceptual framework is put forward for the dissection of biased signaling. This framework provides new insights on biased signaling and novel quantitative scales for system bias, ligand bias, and signaling bias.

To sum up, the new mathematical models and framework are based on some existing operational models for GPCR signaling which have been widely applied to the study of drug action. Therefore, it is feasible to use the rationale and computational tools shown in the present thesis to overcome the difficulties in data analysis which are caused by GPCR oligomerization and biased signaling.

List of articles in this thesis

Bin Zhou, Jesús Giraldo. (2018). Quantifying the allosteric interactions within a G-protein-coupled receptor heterodimer. Drug Discovery Today. 23(1):7-11.

Bin Zhou, Jesús Giraldo. (2018). An operational model for GPCR homodimers and its application in the analysis of biased signaling. Drug Discovery Today. 23(9):1591-1595.

Bin Zhou, David A. Hall, Jesús Giraldo. Biased signaling: covering the whole pharmacological space. Manuscript under review.

Jesús Giraldo, Jordi Ortiz, James Dalton, Bin Zhou. (2017). Chapter 5: Examining Allosterism in a Dimeric G-Protein-Coupled Receptor Context. In Book "Allosterism in Drug Discovery". Published by the Royal Society of Chemistry. (*This publication is not an explicit part of this thesis*).

List of abbreviations

AA:	arachidonic acid
AIAs:	allosteric inverse agonists
ANAs:	allosteric neutral antagonists
ATP:	adenosine triphosphate
cAMP:	adenosine 3,5-monophosphate
DAG:	diacylglycerol
EGFR:	epidermal growth factor receptor
ERK1/2:	extracellular signal–regulated kinase 1/2
GABA:	γ-aminobutyric acid
$GABA_BR$:	γ-aminobutyric acid type B receptor
GDP:	guanosine diphosphate
GPCR:	G-protein-coupled receptor
GTP:	guanosine triphosphate
HB-EGF:	heparin-binding EGF-like growth factor
IL-8:	interleukin-8
IP:	inositol phosphate
IP3:	inositol 1,4,5-trisphosphate
LARG:	leukaemia-associated RhoGEF
LH:	luteinizing hormone
LHR:	luteinizing hormone receptor

LPA:	lysophosphatidic acid
MAP Kinases:	mitogen-activated protein kinases
mGluRs:	metabotropic glutamate receptors
MMP:	matrix metalloproteinase
NAMs:	negative allosteric modulators
PAMs:	positive allosteric modulators
PDEs:	phosphodiesterases
PI3K:	phosphatidylinositol-4,5-bisphosphate 3-kinase
PIP2:	phosphatidylinositol 4,5-bisphosphate
PKC:	protein kinase C
PLC-β:	phospholipase C-β
PP2A:	protein phosphatase 2A
RhoGEF:	Rho guanine-nucleotide-exchange factors
SAMs:	silent allosteric modulators
TRP:	transient receptor potential
TSH:	thyroid stimulating hormone

1. Introduction

In essence, the human body is composed of a great variety of molecules. Generally, they are divided into two categories, namely macromolecules and small molecules. Although this classification is not always suitable, it can help us to dissect an enormous number of molecular interactions. Of the macromolecules, proteins play very important roles in a wide range of biological processes in the human body. For example, some proteins can recognize ligands and then modulate various signaling pathways, causing remarkable changes in biological functions. These proteins are known as receptors. As a consequence, the ligand-receptor interactions have extraordinary implications in the normal physiological processes. Accordingly, it can be anticipated that the disturbance of normal ligand-receptor interactions contributes to a great variety of diseases.

Due to the involvement of abnormal ligand-receptor interactions in plenty of diseases, both the academia and the pharmaceutical industry have paid great attention to the development of drugs which can modulate the abnormal ligand-receptor interactions and restore normal receptor functions. As a large group of receptors, G-protein-coupled receptors (GPCRs) primarily reside in the cell membrane. GPCRs are integral to the normal functions of the cell membrane and responsible for the recognition of extracellular ligands. They assist the cell in detecting external stimuli and sensing the outer world (Smith et al., 2018). In response to the

alterations in the surroundings, the cell subsequently adopts some strategies so as to get adapted to the ever-changing environment.

In the signal transduction processes mediated by GPCRs, these receptors may act on some proteins, which can transduce the signal from the environment into the cell. These proteins downstream of GPCRs are referred to as the transducers. These transducers then interact with the effector molecules, which can further transmit the signal and exert effects on downstream signaling pathways. Not only G proteins, but also β-arrestins or other molecules can transduce the signal mediated by GPCRs (Smith et al., 2018). Discrepant transducers implement their functions by means of different mechanisms of action, which lead to differing responses of the downstream signaling pathways. For some GPCRs, the same receptor can interact with several different transducers. When a particular ligand acts on the receptor, the resultant receptor conformations may interact with some transducers to distinct degrees, causing the differential activation of several downstream signaling pathways. This phenomenon is named “biased signaling”, which is a very hot topic in the domain of pharmacology (Kenakin and Christopoulos, 2013; Smith et al., 2018). The research on biased signaling can help us to circumvent the adverse drug reactions and potentiate the therapeutic efficacy of the drugs.

It is increasingly evident that GPCRs often form oligomers (Ferré et al., 2014; Gomes et al., 2016; González-Maeso, 2014). The oligomerization provides GPCRs with more opportunities to behavior differently from the monomer state (Ciruela et al., 2012). On one hand, a particular GPCR might form a homomer with the same receptor. On the other hand, it is possible that the GPCR can form a heteromer with different receptors. Both homomerization and heteromerization have been observed in experiments, which can assign new functions to GPCRs compared with monomers. GPCR oligomerization is believed to play important roles in normal

physiological processes and a variety of diseases (Borroto-Escuela et al., 2017; Ferré et al., 2014; Gaitonde and González-Maeso, 2017).

Mathematical modeling assists in the analysis of GPCR functions (Roche et al., 2014). The mathematical models can depict how the receptor responds to extracellular ligands and elicits intracellular signal transduction. With the accumulation of evidence that GPCRs exert their functions by means of a range of new mechanisms, an increasing number of mathematical models were developed to address the new modes of action of GPCRs. For instance, the two-state dimer receptor model was constructed to explain the behavior of GPCR homodimers (Franco et al., 2006). Subsequently, a three-state dimer model was built to dissect two different pathways mediated by the same receptor homodimer by assuming that the two active states of the homodimer act on two pathways, respectively (Brea et al., 2009). The asymmetric/symmetric three-state dimer model further presumed that the two active states are the asymmetric R*R state and the symmetric R*R* state, which mediate the G-protein-dependent pathway and the G-protein-independent pathway, respectively (Rovira et al., 2010). This model explained how ligand concentration caused the switch from G-protein-dependent to G-protein-independent signaling for β_2-adrenoceptors.

In addition to the aforementioned models, some operational models were also proposed to account for the working mechanism of GPCR signaling. The Black & Leff model is the first operational model for GPCR signaling, which aims to analyze the agonism of a GPCR monomer (Black and Leff, 1983). Afterwards, the Slack & Hall model was proposed as an extension of the Black & Leff model by taking the constitutive receptor activity into consideration (Slack and Hall, 2012). The addition of the constitutive receptor activity into the model enables the Slack & Hall model to dissect the behavior of inverse agonists. As a result, the Slack & Hall model has the

potential to be applied to the analysis of all types of ligands, including agonists, neutral antagonists, and inverse agonists. However, there were no operational models for GPCR oligomers before the doctoral study of the author of the present thesis.

Some quantitative scales have been proposed to evaluate ligand bias in drug screening programs, which can help us to find the ligands with desirable properties in terms of biased agonism. In some cases, biased ligands are desired, whereas unbiased ligands are preferred under other circumstances. Our preference for biased or unbiased ligands is dependent on the specific situations. Most of the scales defined to date are just applicable to the signaling system without constitutive receptor activity, so they cannot be used for the analysis of inverse agonists (Burgueño et al., 2017; Kenakin and Christopoulos, 2013; Kenakin et al., 2012). A scale for ligand bias was recently proposed by Hall and Giraldo (Hall and Giraldo, 2018). This scale is based on the Slack & Hall model, which can depict the signaling system with constitutive receptor activity. This scale can be employed to analyze the ligand bias of inverse agonists when there is no significant discrepancy in the equilibrium dissociation constant of the ligand-receptor complex between the different signaling pathways. Given the shortcomings of these previous scales, we tried to come up with a new scale which is able to describe the ligand bias of all classes of ligands, including agonists, neutral antagonists, and inverse agonists, without the requirement that the equilibrium dissociation constant of the ligand-receptor complex does not differ significantly between the studied signaling pathways.

There are three studies in the present doctoral thesis. The first study aims at building a new mathematical model for the GPCR heterodimer. The second focuses on a new mathematical model for the GPCR homodimer. The third seeks to propose new quantitative scales for system bias, ligand bias, and signaling bias within a novel conceptual framework for biased signaling.

These three studies assist us in the mathematical modeling of GPCR oligomerization and biased signaling. These mathematical models provide key insights at the functional level on how the signaling systems involving GPCRs interact with the ligands of these receptors. Under some conditions, these models can be employed to fit the experimental data. In these cases, the values of some important parameters in the mathematical models, such as the intrinsic efficacy of the ligand and the cooperativity between ligands, have the potential to evaluate whether a particular ligand is suitable to be further developed in a drug discovery program.

2. Background

2.1. GPCRs

2.1.1. A brief introduction to the GPCRs

GPCRs compose a superfamily of receptors in the biological membrane. The discovery of GPCRs was derived from the research which was done by Martín Rodbell and Alfred G. Gilman. The former investigated how the activity of glucagon peptide is associated with the guanosine triphosphate (GTP) (Rodbell et al., 1971a, 1971b). The latter made parallel observations in adrenergic receptors, and G protein became the name of the protein mediating the signal transduction (Gilman, 1987). Because of these findings, they were awarded the Nobel Prize in 1994.

Subsequently, an increasing number of GPCRs were discovered. Up to now, it is estimated that more than 800 GPCR sequences are located in the human genome (Fredriksson et al., 2003). GPCRs are characterized by seven transmembrane domains which are α-helices. The N-terminus and the C-terminus of a GPCR in the cell membrane are outside and inside the cell, respectively. It has three extracellular loops and three intracellular loops. The ligand binds to the extracellular domain of the receptor and causes the changes in receptor conformations, which elicit alterations in the states of downstream transducers. GPCRs respond to many types

of ligands, such as small molecules and peptides, and the transducers could be G proteins, β-arrestins, or other signaling molecules. The difference in ligands and transducers leads to the large diversity of biological processes influenced by GPCRs.

2.1.2. The classification of the GPCR superfamily

There are a great variety of proteins with a wide range of functions in the GPCR superfamily. In order to gain a better understanding of this superfamily, several classification systems were proposed for dividing the GPCRs into different groups. One of the classification systems uses clans (or classes) A, B, C, D, E, and F (Attwood and Findlay, 1994; Kolakowski, 1994). The A-F system is used for all GPCRs which exist in invertebrates and vertebrates. However, due to the remarkable difference in the types of GPCRs between distant species, this A-F system is not very suitable for the dissection of human GPCRs. Afterwards, the GRAFS classification system was come up with so as to investigate human GPCRs (Fredriksson et al., 2003). According to the GRAFS classification system, the GPCR superfamily in the human genome can be categorized into five primary families, that is, glutamate, rhodopsin, adhesion, frizzled/taste2, and secretin (Fredriksson et al., 2003). It was suggested that the human GPCRs stem from the same ancestor based on the analysis of some shared structural characteristics.

The rhodopsin family contains the largest number of proteins among the five families. The rhodopsin family is consistent with clan A of the A-F classification system. It is worth mentioning that aminergic receptors are in the rhodopsin family, and the aminergic receptors contain some important neurotransmitter receptors, such as 5-hydroxytryptamine receptors, acetylcholine receptors (muscarinic), adrenoceptors, and dopamine receptors (Pándy-Szekeres et al., 2018).

2.1.3. The structures of the GPCRs

The first crystal structure of a GPCR was reported in 2000, when Palczweski et al. determined the three-dimensional structure of rhodopsin from diffraction data (Bourne and Meng, 2000; Palczewski et al., 2000). After that, the crystal structure of the human β_2 adrenergic receptor was published in 2007 (Cherezov et al., 2007; Ranganathan, 2007; Rasmussen et al., 2007; Sprang, 2007). Since then, a growing number of three-dimensional structures of GPCRs have been revealed.

So far, the atomic structures of more than 200 GPCR structures have been determined (Thal et al., 2018). These structures belong to over 50 unique GPCRs and cover various conformational states. These conformations include inactive states, active states, and intermediate states. Some of the GPCRs are in combination with arrestins or G proteins. These GPCR structures provide great insights into the molecular mechanisms of GPCR activation by the extracellular ligands.

2.1.4. GPCRs as drug targets

GPCRs are very versatile in the normal physiological processes. As a consequence, GPCRs are involved in the genesis and development of a great variety of diseases. Additionally, most GPCRs are located in the cell membrane, so the ligands of GPCRs do not need to cross the cell membrane. This phenomenon makes it relatively easy to design drugs which target GPCRs. Due

to these factors, a large number of approved drugs in the market exert their effects by acting through GPCRs.

Among all of the drugs which have been approved by the US Food and Drug Administration (FDA), approximately 34% (475 drugs) target GPCRs (Hauser et al., 2017). Moreover, now there are about 321 agents in clinical trials which have GPCRs as their targets (Hauser et al., 2017). Traditionally, GPCR-related drugs are used for the treatment of schizophrenia, allergy, depression, hypertension, analgesics, and so forth (Hauser et al., 2017). However, GPCR-related ligands are increasingly utilized in the domains of obesity, smoking cessation, Alzheimer disease, hypocalcaemia, multiple sclerosis, and so on.

2.2. G proteins

2.2.1. A brief introduction to G proteins

The full name of G proteins is guanine nucleotide-binding proteins, which belong to the class of enzymes named GTPases. They work as molecular switches inside the cell, whose activity is governed by the conversion between guanosine diphosphate (GDP) and guanosine triphosphate (GTP) bound to them. GTP activates the G protein, while GDP inactivates the G protein. There exist two categories of G proteins, that is, monomeric small GTPases and heterotrimeric G protein complexes. The heterotrimeric G protein complex is constituted by α, β, and γ subunits. The heterotrimeric G proteins can be activated by GPCRs. Depending on the sequence of the α subunit, the heterotrimeric G proteins can be categorized into four

subclasses, namely G_s, $G_{i/o}$, $G_{q/11}$, and $G_{12/13}$. The four subclasses are responsible for mediating the signal from a very wide range of extracellular ligands. Figure 1 shows some examples of signaling mediated by G proteins.

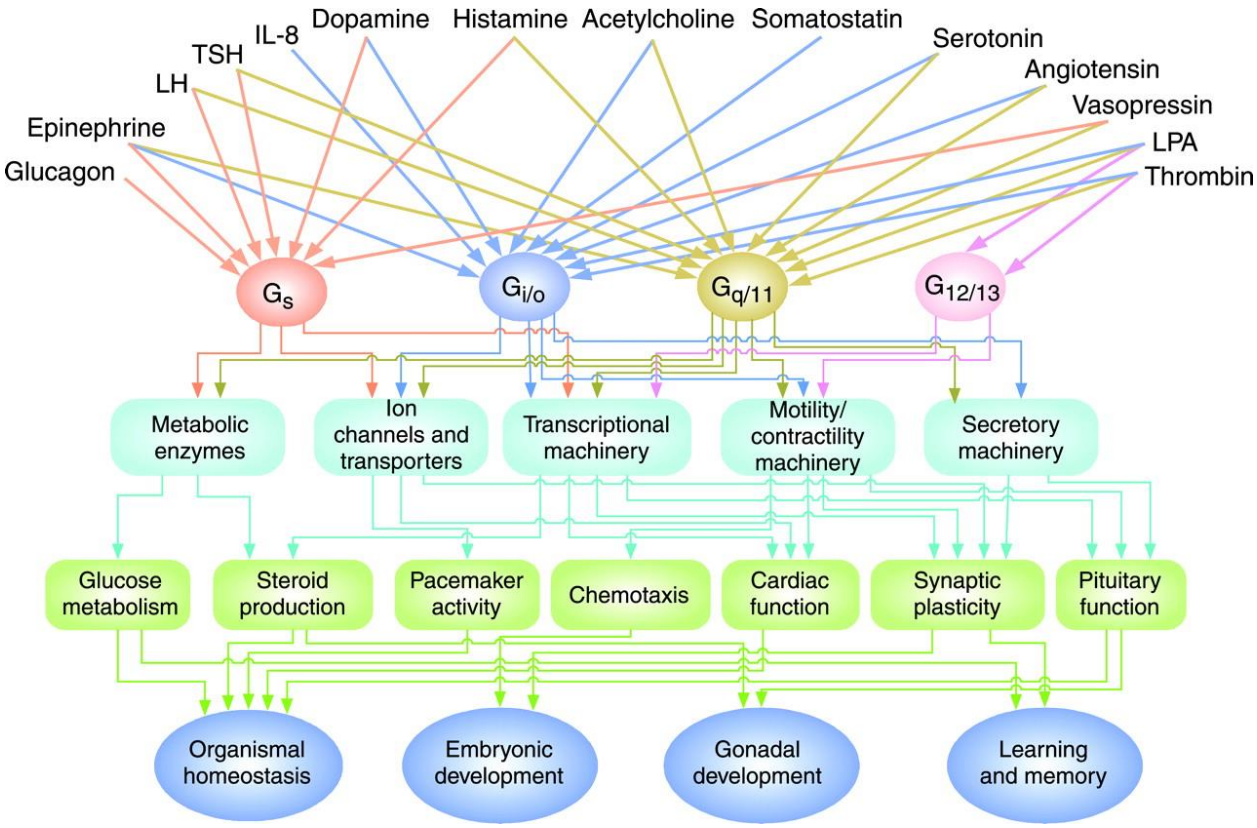

Figure 1. Modulation of systemic functions by four subclasses of G proteins. The extracellular stimuli include neurotransmitters, hormones, chemokines, and so forth. These ligands interact with a variety of GPCRs and thus cause the functional changes in the four subclasses of G proteins. Of note, some ligands can act at more than one GPCR and regulate over one G protein. Subsequently, G proteins affect the functional state of some cellular machines. The cellular machines further modulate cellular functions, eventually resulting in the alteration of systemic functions. LH: luteinizing hormone; TSH: thyroid stimulating hormone; IL-8: interleukin-8; LPA: Lysophosphatidic acid. This picture has been taken from the article of Neves et al. (Neves et al., 2002).

2.2.2. $G\alpha_s$ and downstream signaling pathways

$G\alpha_s$ can activate the adenylate cyclase, which turns the adenosine triphosphate (ATP) into the adenosine 3,5-monophosphate (cAMP) (Bourne, 1997; Tesmer et al., 1997). cAMP can stimulate cAMP-dependent protein kinases, thereby playing a role in the regulation of a vast array of cellular responses.

2.2.3. $G\alpha_{i/o}$ and downstream signaling pathways

By means of the inhibitory effect of this group of G protein subunits on the adenylate cyclase, the production of cAMP is reduced, which leads to the weaker function of cAMP-dependent protein kinases (Dessauer et al., 2002). In this manner, these G protein subunits can hinder the cAMP-dependent pathways.

2.2.4. $G\alpha_{q/11}$ and downstream signaling pathways

This group of G protein subunits can activate the phospholipase C-β (PLC-β) (Neves et al., 2002). In the next step, PLC-β hydrolyzes the phosphatidylinositol 4,5-bisphosphate (PIP2) and thus generates the diacylglycerol (DAG) and the inositol 1,4,5-trisphosphate (IP3) (Kamato et al., 2017). Subsequently, DAG stays on the membrane and activates the protein kinase C (PKC). At the same time, IP3 goes to interact with IP3 receptors in the endoplasmic reticulum, resulting in the accumulation of calcium in the cytosol. These processes elicit a variety of alterations in cellular functions.

2.2.5. $G\alpha_{12/13}$ and downstream signaling pathways

These G protein subunits can stimulate Rho guanine-nucleotide-exchange factors (RhoGEF), such as leukaemia-associated RhoGEF (LARG) in mammals (Worzfeld et al., 2008). This action will cause the activation of RhoA. In addition, this group of G protein subunits can also interact with other molecules. For instance, $G\alpha_{12/13}$ can prevent cadherins from exerting adhesive effects and cause the β-catenin to be released (Meigs et al., 2001, 2002).

2.3. β-arrestins

2.3.1. A brief introduction to β-arrestins

Arrestins constitute a small protein family playing crucial roles in the modulation of signal transduction (Lefkowitz and Shenoy, 2005; Moore et al., 2007). There are four subtypes of arrestins in mammals, namely arrestin-1 (visual arrestin), arrestin-2 (β-arrestin1), arrestin-3 (β-arrestin2), and arrestin-4 (cone arrestin) (Smith and Rajagopal, 2016). Among them, arrestin-1 and arrestin-4 are expressed in the eyes, whereas arrestin-2 and arrestin-3 are ubiquitously expressed (Smith and Rajagopal, 2016). Arrestin-2 and arrestin-3 belong to β-arrestins. At the beginning, β-arrestins were found to induce the desensitization of β_2 adrenergic receptor after it was activated by the agonist, but until now it has been revealed that β-arrestins have a wide range of functions in the regulation of biological processes (Smith and Rajagopal, 2016).

2.3.2. β-arrestin-mediated signaling pathways

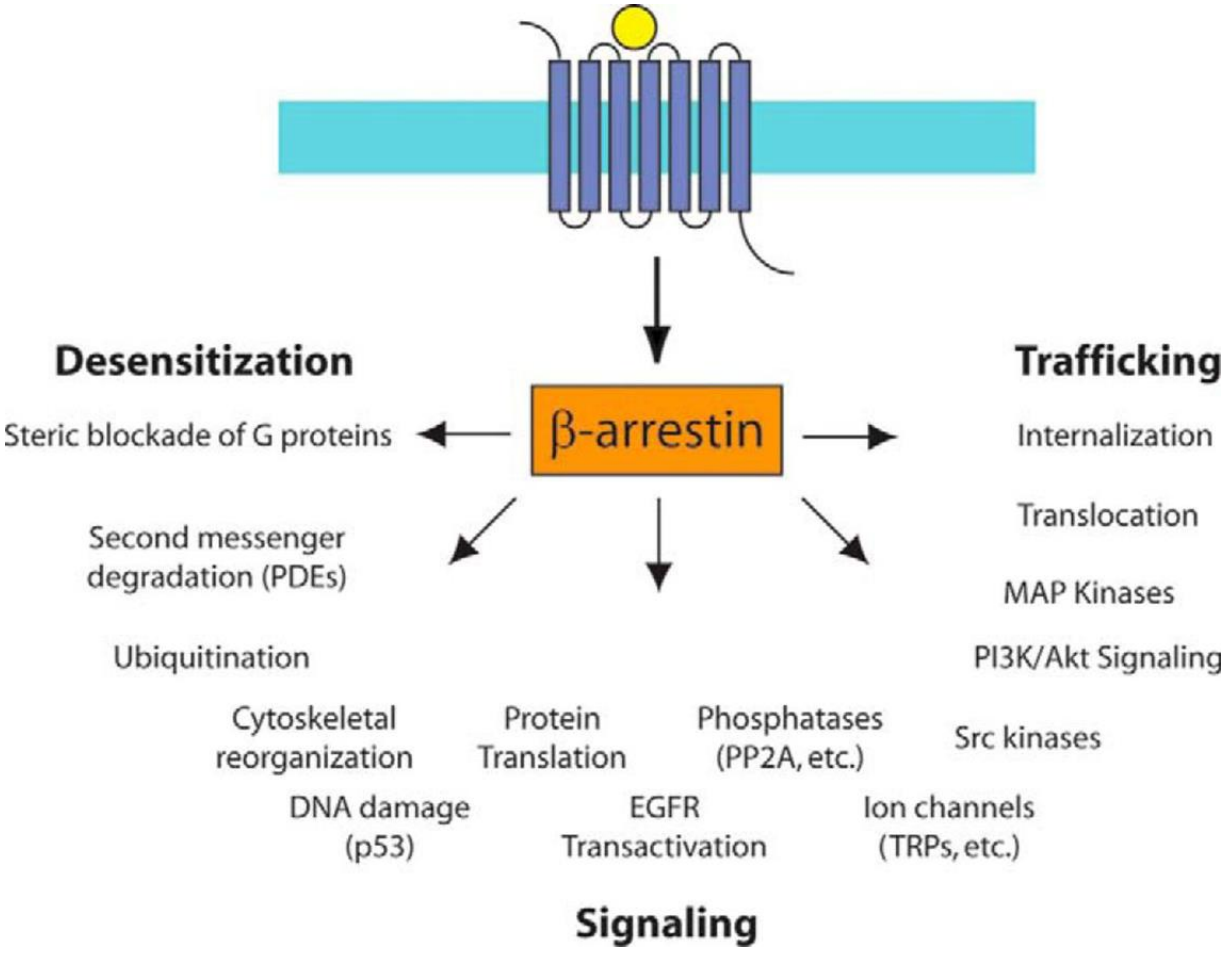

Figure 2. Some examples of the biological functions of β-arrestins. EGFR: epidermal growth factor receptor; MAP Kinases: mitogen-activated protein kinases; PP2A: protein phosphatase 2A; PDEs: phosphodiesterases; PI3K: phosphatidylinositol-4,5-bisphosphate 3-kinase; TRP: transient receptor potential. This picture is from the article of Smith and Rajagopal (Smith and Rajagopal, 2016).

2.4. Mechanisms of signal transduction mediated by GPCRs

2.4.1. The traditional model

There are several models for GPCR functions. The traditional one is that the agonist-bound GPCR in the cell membrane attracts heterotrimeric G proteins and substitute a GTP for a GDP on the G proteins, resulting in the dissociation of their α-subunits from βγ-subunits and thus the activation of both α-subunits and βγ-subunits. The GTP-bound α-subunit then binds to the downstream effectors, causing signal transduction in the cell. Sometimes the βγ-subunit can also bind to some effectors. After a period, the GTP binding to the α-subunit is converted into GDP by hydrolysis. Subsequently, the GDP-bound α-subunit associates with the βγ-subunit again, forming the GDP-bound G protein, which is inactive and waits to bind to a GPCR. This traditional view of GPCR activation is being challenged by numerous new experimental observations.

2.4.2. Intracellular signaling

At the beginning, GPCRs were thought to be located merely in the cell membrane and initiate the signaling cascades from there. Nevertheless, an increasing amount of evidence is showing that GPCRs can also perform their functions inside the cell in addition to working as the detectors of the extracellular ligands in the cell membrane (Luttrell et al., 1999; Schiaffino et al., 1999). GPCRs off the cell membrane can be activated in several ways. One way to achieve activation is that GPCRs can be directly stimulated in some organelles, also known as the internalization-independent intracellular activation. Another way is that GPCRs can be activated after the internalization, which is termed the internalization-dependent activation.

Besides the cell membrane, GPCRs were also reported to exist in the lysosome, the melanosome, the cell nucleus, the endoplasmic reticulum, and the mitochondrion (Bénard et al., 2012; Oksche et al., 2000; Revankar et al., 2005; Rozenfeld and Devi, 2008; Schiaffino et al., 1999; Sergin et al., 2017). As a consequence, the internalization-independent intracellular activation may occur for the GPCRs residing in these locations. For instance, ocular albinism type 1 protein is located in the melanosome rather than the plasma membrane, and it can stimulate G proteins on the cytoplasmic side (Schiaffino et al., 1999). In this way, it plays a role in the organelle biogenesis and maturation. One of other examples is the metabotropic glutamate receptor 5. The receptor can reside in the inner nuclear membrane and is responsible for causing the alterations in Ca^{2+} in the nucleoplasm (Sergin et al., 2017).

The β-arrestin performs very important functions in the internalization-dependent activation. It enhances the endocytosis of GPCRs and acts on the MAP kinase pathway (Eichel et al., 2016). The internalization-dependent activation can be implemented in several ways. With respect to class A GPCRs, the β-arrestin can bind to the receptor in the cell membrane. Afterwards, the complex formed by the β-arrestin and the GPCR is internalized, which results in the activation of the extracellular signal–regulated kinase 1/2 (ERK1/2) (Luttrell, 2005).

2.4.3. Pre-coupling of G proteins to GPCRs

There is some evidence showing that G proteins are pre-coupled to inactive GPCRs (Weiss et al., 1996). The binding of agonists affects the state of the macromolecular complex of the GPCR and the G protein, altering the conformations of the G protein. The G protein then becomes active and influences the state of the downstream effector molecules, thus eliciting the intracellular signal transduction.

2.4.4. Transactivation through other receptors

Some GPCRs (such as serotonin receptors and chemokine receptors) are capable of transactivating other receptors (such as pattern recognition receptors and tyrosine kinase receptors) in the membrane (Abdulkhalek et al., 2012; Fischer et al., 2004; Itoh et al., 2005; Kruk et al., 2013). The phenomenon of transactivation makes GPCRs much more versatile in regulating the wide range of physiological processes. One of the well-known instances is that GPCRs can transactivate the epidermal growth factor receptor (EGFR). Figure 3 illustrates how GPCRs can activate the EGFR in three distinct manners.

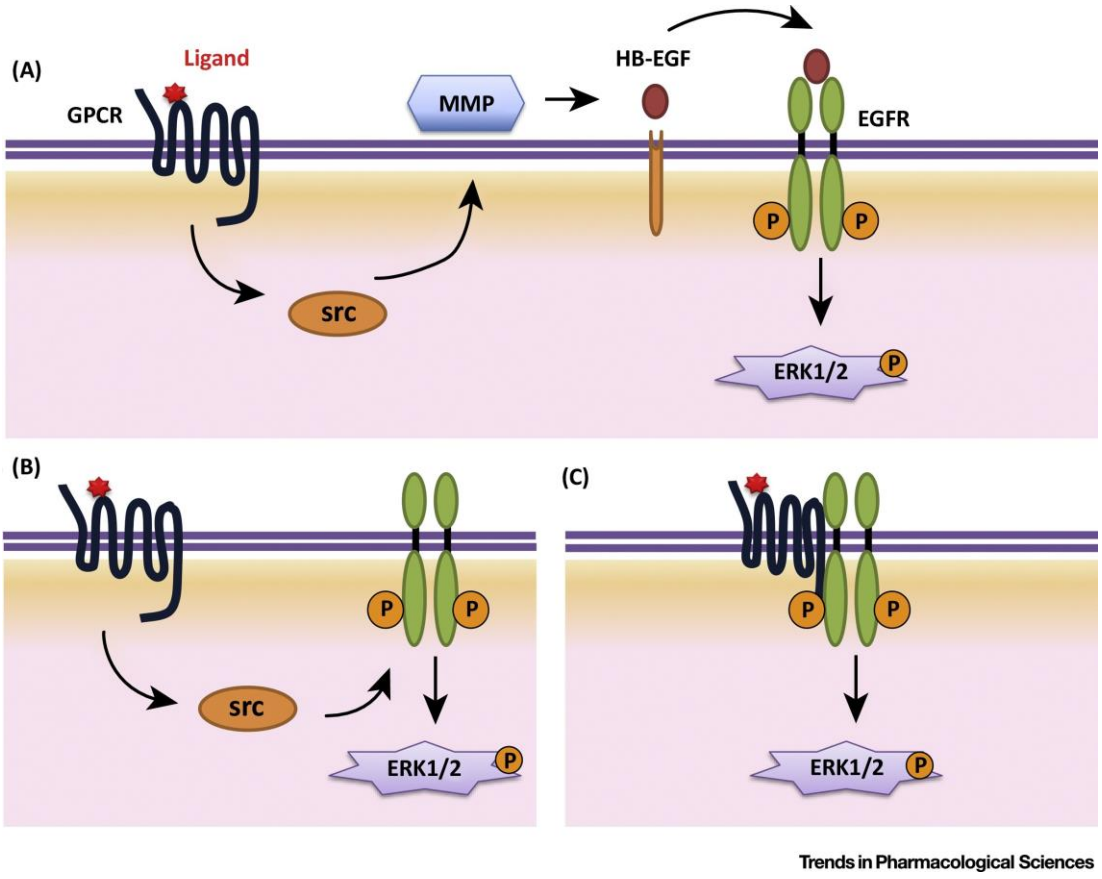

Figure 3. Transactivation of the EGFR by the GPCR. There are three main approaches to accomplishing the transactivation of the EGFR. (A) The GPCR stimulates the src kinase, and the src kinase then promotes the production of the matrix metalloproteinase (MMP). Subsequently, the MMP causes the heparin-binding EGF-like growth factor (HB-EGF) to leave the membrane. In the next step, the HB-EGF activates the EGFR by the direct interaction. (B) The src kinase phosphorylates the EGFR straightly. (C) The GPCR elicits the stimulation of the EGFR by generating a molecular complex in combination with the EGFR. This figure comes from the article of Wang et al. (Wang et al., 2018).

2.4.5. Biased signaling

GPCRs can also transmit the extracellular signal into the cell through β-arrestins or other transducer molecules in addition to G proteins (Smith et al., 2018). The diversity of downstream transducers leads to a phenomenon referred to as "biased signaling". Specifically, biased signaling occurs if the GPCR-ligand complex influences several downstream signaling pathways in different ways.

It is thought that biased signaling has its roots in the multiplicity of active conformations of the receptor. The receptor is always in the equilibrium between these discrepant conformations. Some active conformations are relatively more amenable to a subset of the transducers, while other active conformations may prefer other transducers. Some ligands have the ability to alter the conformational equilibrium, selecting some dominant conformations. By doing so, some ligands are pushing the receptor to some of the transducers, leading to the preferential activation of the corresponding downstream signaling pathways. Provided that a ligand

differentially influences the distinct signaling pathways downstream of a receptor, this ligand is biased towards some of the pathways and it is called a biased ligand.

In addition to biased ligands, biased signaling may also be caused by biased systems. A signaling system is made up of a receptor, some transducers, and some effectors. Owing to the difference in relative expression of these components in a signaling system, the system may be biased towards some of the signaling pathways even in the absence of the ligands. When the signaling system prefers some pathways to others without the addition of the ligands, this system can be thought of as a biased system.

Biased ligands are the objectives of the research in many programs of drug discovery. This is due to the advantages which biased ligands may have in the potentiation of therapeutic effects and the reduction of adverse drug reactions. Traditional drugs possess remarkable side effects. One of the causes of these effects is that the drugs modulate too many signaling pathways in the human body, and some of the affected pathways are responsible for the adverse drug reactions. In order to reduce the adverse drug reactions, it is necessary to develop drugs which can selectively act on a subset of the signaling pathways. By being biased towards some particular pathways, the biased ligands have a more focused mechanism of action, which increases the possibility that the ligands will become approved drugs in the market. Some biased ligands have been discovered for a variety of receptors, such as the dopamine D2 receptor and the μ-opioid receptor.

Many antipsychotics are unbiased antagonists for the dopamine D2 receptor. Their unbiased action can produce extrapyramidal side effects in addition to the antipsychotic effects. Recently, some researchers found the D2 agonists which are biased towards the β-arrestin. These

agonists do not bear the extrapyramidal side effects, but they still retain the antipsychotic functions (Rankovic et al., 2016).

Opioids, especially morphine, often work as analgesics for pain. Morphine acts as an agonist for the μ-opioid receptor and exerts its effects via the β-arrestin and the G protein. It was revealed that the β-arrestin mediates some side effects of morphine, while the G protein plays a role in pain relief (Rankovic et al., 2016). Given the differences in functions between the β-arrestin and the G protein, efforts are being made to develop the drugs which are biased towards the G-protein-dependent pathway. PZM21 is a ligand which was discovered along this research direction (Manglik et al., 2016).

2.5. Oligomerization of GPCRs

The classical view of the GPCR signaling is that these receptors act as monomers to transmit the extracellular signal into the cell. In spite of that, a breakthrough was made by Maggio el al., who found that the ability in binding and signaling of pairs of GPCR fragments or chimeras were restored when they were expressed together and located in the same membrane, although they were in the inactive state if they were produced separately (Maggio et al., 1993, 1996). After that, another study reported that when the signaling-deficient luteinizing hormone receptor (LHR) and the binding-deficient LHR were generated together in the transgenic mice and there were no normal wild-type receptors, the mutant receptors could complement the capability of each other by the interaction between them and thus the luteinizing hormone could still exert its effects (Rivero-Müller et al., 2010; Vassart, 2010). This study revealed the existence of GPCR dimers which are functional in the mice and the influence of receptor dimerization on ligand binding and the coupling of transducers to GPCRs.

With the accumulation of experimental observations, there is growing evidence showing that plenty of GPCRs can also form functional oligomers, although numerous GPCRs can still work in the monomeric style. In the oligomer, each receptor is called a protomer. The oligomer may be a homomer or a heteromer depending on the identity of the protomers. Provided that the protomers are the same receptor, the oligomer is a homomer. On the contrary, the oligomer is a heteromer if the protomers are different.

Up to now, the three-dimensional structures of the homodimers of some GPCRs have been determined, such as the κ-opioid receptor, the μ-opioid receptor, and the CXCR4 chemokine receptor (Manglik et al., 2012; Wu et al., 2010, 2012). These structures provide the support for the existence of GPCR homodimers and assist in the understanding of the molecular mechanisms of GPCR homodimerization.

Oligomerization enables the protomers within an oligomer to communicate with each other. In other words, a protomer can affect the functions of others in an oligomer. The interaction between individual protomers within an oligomer influences not only the binding of the ligands to the receptors but also the coupling of downstream transducers and effectors to the receptors.

γ-aminobutyric acid (GABA) acts as the primary inhibitory neurotransmitter in the central nervous system of the mammals, and the GABA type B receptor ($GABA_BR$) works to mediate the signal from GABA. A good example of the communication between the protomers within an oligomer is the heterodimer consisting of $GABA_BR1$ and $GABA_BR2$ receptors. The heterodimer is fully functional and can respond to GABA, whereas each of the two components cannot

mediate the full signal transduction from the ligand individually in the absence of the other (Kaupmann et al., 1998; Kuner et al., 1999; White et al., 1998).

The aforementioned instance displays the functional significance of the formation of the heteromer. In addition to heteromerization, homomerization also has biological functions. For example, metabotropic glutamate receptors (mGluRs) are constitutive homodimers, and homodimerization is necessary for the signal transduction which is mediated by these receptors.

Owing to the important roles of GPCR oligomerization in a great variety of physiological processes, GPCR oligomers are potential drug targets for a wide range of disorders (Borroto-Escuela et al., 2017). As a consequence, some researchers are trying to develop selective ligands for GPCR oligomers, such as the heterodimer which is composed of dopamine D2 receptor and neurotensin NTS1 receptor (Hübner et al., 2016). The ligands which selectively target GPCR oligomers may be better drugs for some diseases.

2.6. Allostery and cooperativity

Allostery means the mutual influence between distinct sites. In pharmacology, allostery is widely used to describe the interaction between spatially separate sites of proteins which can impact the functions of the proteins. Since extracellular ligands can change the conformations of intracellular domains of GPCRs, allostery is an intrinsic property of GPCRs. In addition to the allosteric interactions between the extracellular and the intracellular domains, there is mutual influence between the orthosteric and the allosteric sites on the extracellular domains of GPCRs. According to the difference in binding sites, the ligands for GPCRs are classified into two categories, that is, orthosteric ligands and allosteric ligands.

The ligands binding to the orthosteric sites are referred to as orthosteric ligands. These ligands could be agonists, neutral antagonists, or inverse agonists depending on how they affect the functions of the receptor. Agonists and inverse agonists have positive and negative effects on the signaling mediated by the receptor, respectively. Neutral antagonists do not change the functions of the receptor. Under some circumstances, antagonists include both neutral antagonists and inverse agonists.

The ligands bound to the allosteric sites are termed allosteric ligands. Some allosteric ligands cannot directly affect the function of the receptor, but can modulate the effect of orthosteric ligands on the receptor. These allosteric ligands are called allosteric modulators. There are three types of allosteric modulators, namely positive allosteric modulators (PAMs), silent allosteric modulators (SAMs), and negative allosteric modulators (NAMs). The PAMs can potentiate the impact of the orthosteric agonist on the receptor, while the NAMs decrease the effect of the orthosteric agonist. The SAMs just occupy the allosteric binding site and do not affect the function of the orthosteric agonist.

In addition, there are also some allosteric ligands that can directly activate the receptor. These allosteric ligands are referred to as allosteric agonists. Allosteric agonists can perform their function even without the orthosteric ligand. By analogy, allosteric inverse agonists (AIAs) and allosteric neutral antagonists (ANAs) are respectively functionally similar to inverse agonists and neutral antagonists except that AIAs and ANAs are bound to the allosteric sites rather than the orthosteric sites.

One frequently observed phenomenon is that there is cooperativity between the different ligands that bind to the GPCRs. In terms of the attribute of the cooperativity, there are two

types of cooperativities, namely the binding cooperativity and the activation cooperativity. On the one hand, provided that a ligand affects the binding of another ligand to the receptor, binding cooperativity exists between the two ligands. On the other hand, there is activation cooperativity between the two ligands which have already been bound to the receptor if a ligand influences the ability of the other ligand to activate or inhibit the receptor.

The cooperativities between ligands originate from the phenomenon of allostery for GPCRs. For example, allosteric communications occur between the allosteric sites and the orthosteric sites of GPCRs, so there are binding and activation cooperativities between the allosteric ligands and the orthosteric ligands. As mentioned in the section of the oligomerization of GPCRs, these receptors often combine together to form oligomers. It was reported that there are also allosteric interactions between the protomers making up an oligomer. Accordingly, it can be anticipated that cooperativities also exist between the ligands which are bound to different protomers in an oligomer. In this way, a ligand can change the functional state of another receptor in addition to its direct target. The existence of cooperativities makes it more complex to regulate the activities of the collection of molecules in the human body. From another perspective of therapeutics, the cooperativities enable us to design the drugs which are capable of affecting the functions of a variety of proteins.

It is widely accepted that the treatment of complex diseases possibly requires the simultaneous targeting of multiple proteins. GPCR oligomerization and the resultant allostery between different GPCRs provide us with an approach to targeting more than one protein using one ligand. Under some conditions, the combination of several drugs which are respectively bound to the different proteins within a GPCR oligomer can be employed to treat a disease. In these cases, the binding and activation cooperativities between these drugs should be taken into

consideration so as to maximize the therapeutic effects and minimize the adverse drug reactions.

2.7. Previous mathematical models of GPCR signaling

2.7.1. An introduction to mathematical modeling of GPCR functions

Mathematics is widely applied to the quantitative description of a great variety of phenomena. It can give us the equations for calculating the parameter values for the relevant properties of the system. Accordingly, mathematical models assist us in the understanding of almost all of the events which are happening. In the field of pharmacology, mathematical models are mainly intended to depict how drugs elicit the functional responses of various signaling systems. The correlation between the functional responses and the drug concentration can be displayed by some equations. According to the sources of the equations, the mathematical models of GPCR signaling can be categorized into three classes, namely mechanistic models, empirical models, and hybrid models.

The parameters in mechanistic models are physicochemical constants which are used to delineate the physicochemical processes, such as the process of the binding of the ligand to the receptor. The empirical models aim at the description of the shapes of functional response curves and the quantitative association between the functional response and the concentration of the ligand. The parameters in the empirical models are just descriptors of the curves and do not have physicochemical meanings. Hybrid models are the combinations of mechanistic models and empirical models. Some of the parameters in the hybrid models are physicochemical constants, while other parameters are not. The present doctoral thesis focuses

on operational models (a type of hybrid models). In this Background section, some previously proposed mechanistic models and operational models are discussed. They provide the foundation for the development of the new mathematical models of GPCR signaling.

2.7.2. The two-state model of receptor activation

Figure 4 shows the two-state model of receptor activation (Leff, 1995). There are two states (one inactive state and one active state) for the receptor. R and AR are in the inactive state, while R* and AR* are in the active state. A is the ligand which binds to the receptor. K_A and K_A* are the equilibrium dissociation constants for AR and AR*, respectively. L is the equilibrium transformation constant between R and R*. The three parameters are defined by the following equations.

$K_A = [A] [R] / [AR]$

$K_A^* = [A] [R^*] / [AR^*]$

$L = [R] / [R^*]$

The total receptor concentration $[R]_{total} = [R] + [AR] + [R^*] + [AR^*]$

The fractional functional response $f = E / E_m$, where E denotes the absolute functional response of the signaling system and E_m represents the maximum possible functional response of the signaling system. It is assumed that the fractional functional response f is equal to the percentage of the active receptors among total receptors: $f = ([R^*] + [AR^*]) / [R]_{total}$.

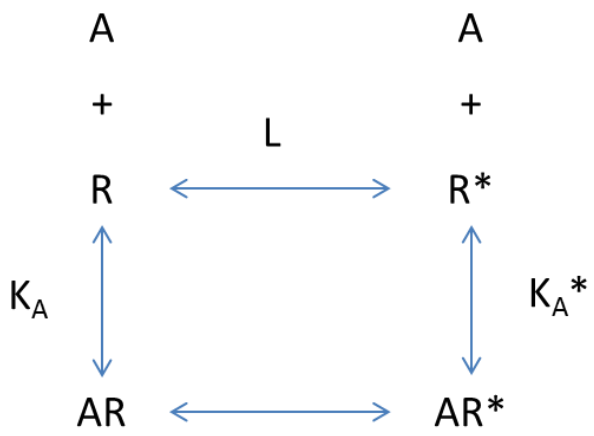

Figure 4. The two-state model of receptor activation.

According to the model, the type of ligands depends on the comparison between K_A and K_A*. For the agonists, K_A is greater than K_A*. On the contrary, K_A is less than K_A* for the inverse agonists. Regarding the neutral antagonists, K_A is equal to K_A*. In other words, the agonists prefer R* to R, whereas the inverse agonists favor R relative to R*. The basal functional response of the system is determined by the parameter L.

This model has some merits. For example, it can account for the constitutive receptor activity and distinguish between three types of ligands, namely agonists, neutral antagonists, and inverse agonists. Nevertheless, it is intended for the receptor monomer and takes into consideration only two states of the receptor. These limitations make this model unable to address some new problems, such as GPCR oligomerization. Therefore, the following mathematical models were subsequently proposed to depict the new phenomena of GPCR oligomerization and biased signaling.

2.7.3. The two-state dimer receptor model

Figure 5 illustrates the two-state dimer receptor model (Franco et al., 2006). This model thinks of the receptor homodimer as an entire functional entity. This homodimer has two sites which can bind to the ligands. There are two states (one inactive state and one active state) for this entity. The active state can stimulate the downstream signaling pathway mediated by this homodimer. The ligand influences the percentage of active homodimers via conformation induction or selection. In this model, (R_2) and (R_2)* represent the inactive homodimer and the active homodimer, respectively. A symbolizes the ligand which is bound to the homodimer. L is the equilibrium transformation constant which describes the constitutive receptor activity of the homodimer. K is the equilibrium association constant for the binding of the ligand to the free homodimer. α and β mean the intrinsic efficacies of the first ligand and the second ligand for the receptor homodimer, respectively. Consequently, β/α is the activation cooperativity between the two ligands. In the circumstance where β/α = 1, the first ligand does not have the impact on the function of the second ligand. Under this condition, μ is the binding cooperativity between the two ligands.

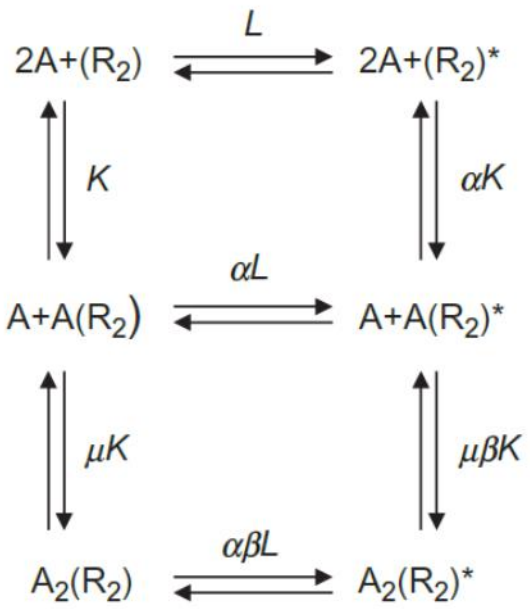

Figure 5. The two-state dimer receptor model.

The two-state dimer receptor model takes the GPCR homodimerization into account and has the ability to explain the influence of the homodimerization on the ligand-receptor interactions. Nevertheless, the receptor homodimer in this model has just one inactive state and one active state. As a consequence, this model cannot calculate more than one fractional functional response at the same time. It means that this model cannot be used to analyze two or more signaling pathways which have different fractional functional responses. Given this drawback of the model, the three-state dimer model was put forward to account for the distinct fractional functional responses which may be achieved by several signaling pathways downstream of the receptor homodimer.

2.7.4. The three-state dimer receptor model

The three-state dimer receptor model was proposed by Brea et al. to explain how distinct signaling pathways are mediated by the same receptor homodimers. This model can delineate the interactions between some ligands and the serotonin 2A receptor in a pathway-specific way. In this model, the receptor homodimer may be in one inactive state (R_2) or two discrepant active states [(R_2)* and (R_2)**]. In the experimental observations, (R_2)* may be related to the IP (inositol phosphate) accumulation pathway, while (R_2)** may mediate the AA (arachidonic acid) release pathway. This model is an extended version of the two-state dimer receptor model which is mentioned before. Because there are two active states in the three-state dimer receptor model, the model can give the values of two fractional functional responses, and each response can be from one of the two downstream signaling pathways.

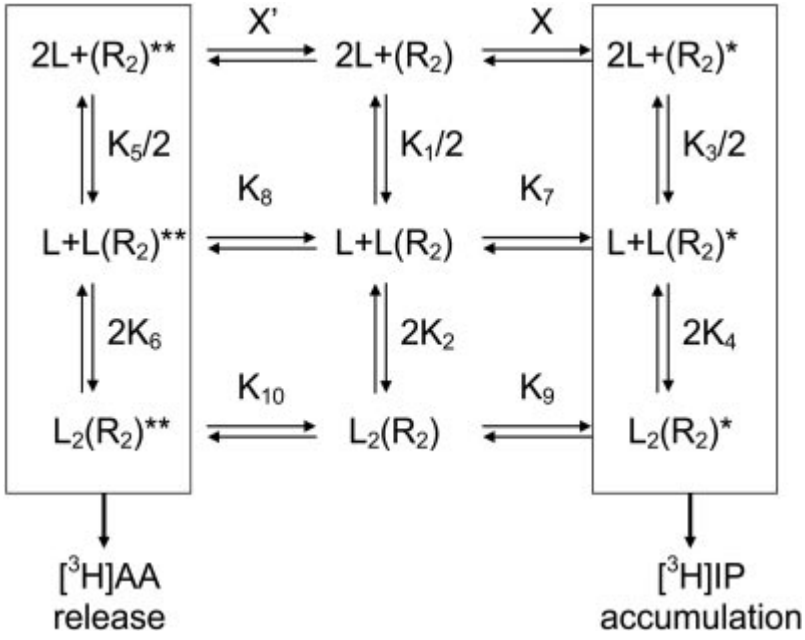

Figure 6. The three-state dimer receptor model (Brea et al., 2009). X, X', K_7, K_8, K_9, and K_{10} are equilibrium transformation constants. K_1, K_2, K_3, K_4, K_5, and K_6 are equilibrium dissociation constants.

2.7.5. The asymmetric/symmetric three-state dimer model

The aforementioned three-state dimer receptor model views the receptor homodimer as a global entity, whereas the asymmetric/symmetric three-state dimer model differentiates between the two protomers within a receptor homodimer. The latter model postulates how the three states of the receptor homodimer are determined by the states of the individual protomers. Specifically, when both protomers are inactive, the homodimer is in the inactive state. If one of the two protomers is inactive and the other is active, the homodimer is in the asymmetric active state. When both protomers are active, the homodimer is in the symmetric active state. According to the experimental observations, the asymmetric/symmetric three-state dimer model infers that the asymmetric active state is correlated with the signaling pathways which are mediated by G proteins, while the symmetric active state is related to the signaling pathways which are mediated by other proteins.

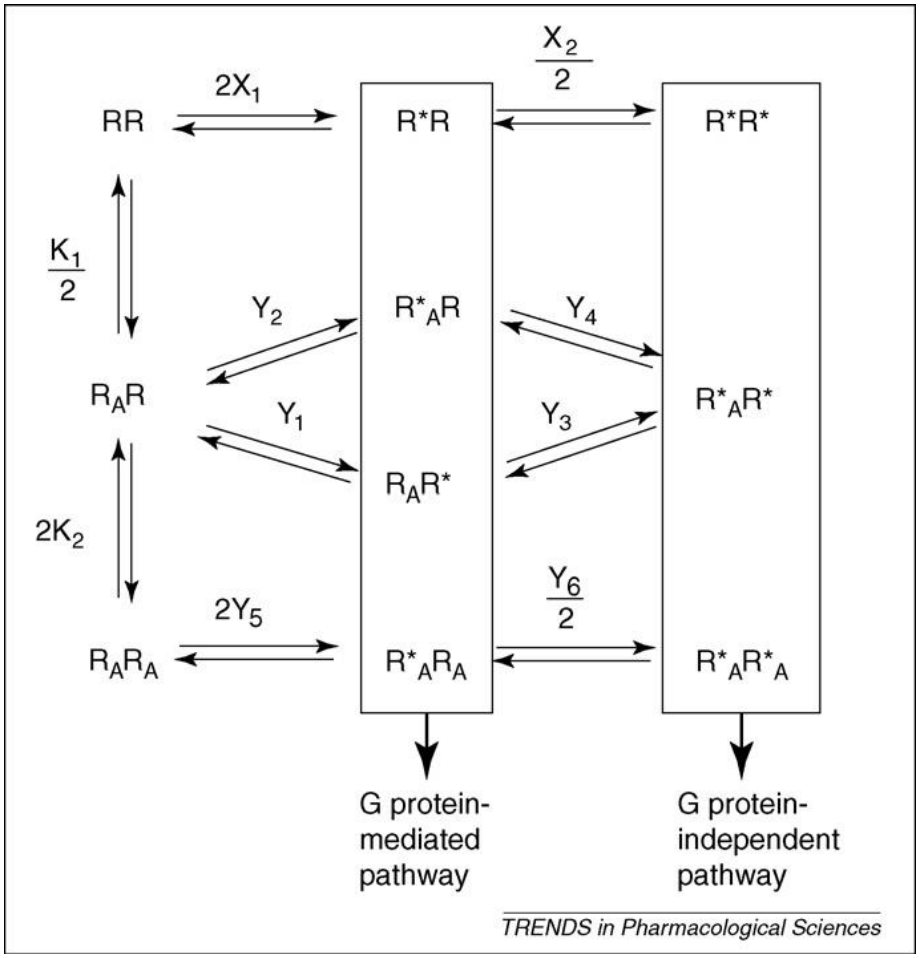

Figure 7. The asymmetric/symmetric three-state dimer model (Rovira et al., 2010). The symbol A represents the ligand which can bind to the receptor. R and R* are the inactive and the active protomers, respectively. The parameters are the equilibrium constants for the corresponding processes.

2.7.6. The operational model of agonism (the Black & Leff model)

The operational model of agonism describes the receptor signaling as a two-step process (Black and Leff, 1983). The first step is that the ligand binds to the receptor. In the second step, the

ligand-receptor complex stimulates the downstream signaling pathways. An empirical function is proposed for the transduction of receptor occupancy into effect (see Figure 8A).

2.7.7. The operational model of agonism with constitutive receptor activity (the Slack & Hall model)

The Slack & Hall model is an extended version of the operational model of agonism by taking the constitutive receptor activity into account (Slack and Hall, 2012). In the Slack & Hall model, both the free receptor and the ligand-receptor complex can activate the downstream signaling pathways. Therefore, this model defines the new term stimulus. The stimulus is made up of two parts, one from the free receptor and the other from the ligand-receptor complex. Because the free receptor and the ligand-receptor complex may differ in the ability to generate the stimulus, distinct weights are assigned to the free receptor and the ligand-receptor complex in the composition of the stimulus. When the weight of the free receptor is 1, the weight of the ligand-receptor complex is the intrinsic efficacy of the ligand, which means the ability of the ligand to activate the downstream signaling pathways.

The intrinsic efficacy is a very important parameter. It can be used to distinguish between different types of ligands, such as agonists, neutral antagonists, and inverse agonists. As a consequence, the Slack & Hall model can explain the behavior of the three types of ligands.

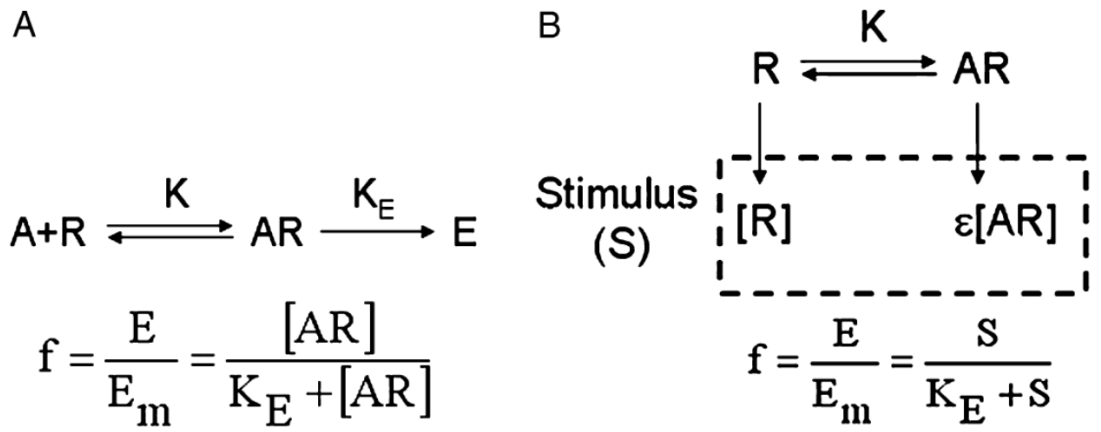

Figure 8. The operational model of ligand action. (A) The operational model of agonism (the Black & Leff model). (B) The operational model of agonism with constitutive receptor activity (the Slack & Hall model). In both models, A, R, and AR denote the ligand, the receptor, and the ligand-receptor complex, respectively. K is the equilibrium dissociation constant. E means the absolute functional response, whereas E_m represents the maximum possible functional response of the signaling system. f symbolizes the fractional functional response. S is the stimulus which elicits the functional response. ε is the intrinsic efficacy of the ligand. When f = 0.5, K_E is equal to [AR] in the Black & Leff model or S in the Slack & Hall model.

3. Objective

The objective of the present thesis is to provide mathematical models and the framework for the understanding of some new concepts in the research on the signaling mediated by GPCRs, particularly GPCR oligomerization and biased signaling. There are three studies in the present thesis. The goals of the three studies are listed as follows.

- Propose a quantitative mathematical model for GPCR heterodimers.

- Come up with a new mathematical model for GPCR homodimers.

- Build a new conceptual framework for biased signaling and put forward new quantitative scales for system bias, ligand bias, and signaling bias based on this framework.

4. Publications

4.1. The first work

Quantifying the allosteric interactions within a G protein-coupled receptor heterodimer

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Keywords: GPCR; heterodimer; operational model; receptor crosstalk; cooperativity; signaling pathway

Teaser: A mathematical model for a receptor heterodimer is presented which quantifies receptor crosstalk and the ligand-dependent dominance of one receptor over the other. The model can be useful for a mechanistic design of drug combinations

Abstract

G protein-coupled receptors are central to signal transduction and cell communication. The possibility that cells use receptor heteromerization as a way to modulate individual receptor pathways is a surmise that cannot be precluded. Given the complexity of these processes, mathematical models are of great help in understanding how receptors and their respective ligands regulate signaling. Here, a mathematical model is presented which quantifies the allosteric interactions within a receptor heterodimer. The model is based on the operational model of allosterism including constitutive receptor activity, which provides the pharmacological analysis of heteromerization with well-established and widely-used modeling and fitting procedures.

Introduction

G protein-coupled receptors (GPCRs) are a superfamily of membrane receptors which mediate multiple signaling pathways in living organisms. They exist in the cell membrane and connect the signals outside the cell with the change in biological processes inside the cell. Due to the involvement of these proteins in many diseases, there has been much research on the mechanisms underlying GPCR function and on drugs targeting GPCRs [1]. However, it remains unclear how drugs impact the receptors and thus cause their functional effects.

It was traditionally thought that GPCRs act as monomers, but now increasing evidence shows that they may interact with each other to form dimers and higher-order oligomers [2]. Heteromerization, i.e. the physical combination of different receptor proteins into a new receptor entity, establishes the foundation for direct crosstalk between signaling pathways respectively mediated by these proteins. In this manner, one single ligand can induce alterations in various cellular processes. GPCR heteromerization has been postulated for a wide range of receptors [3-6] and is thought to be related to various neurologic and neuropsychiatric disorders [2,7], including schizophrenia [8], tardive dyskinesia [9] and opioid use disorders [10] amongst others. Therefore, developing new treatments for these conditions would require a thorough understanding of heteromerization. Moreover, heteromerization has the potential to be exploited for the development of more potent therapies with fewer side effects by utilizing

synergistic drug combinations. Finally, while heteromerization enables the cell to make full use of GPCR signaling, the complexity in data analysis poses a great challenge to the scientific research into GPCR function and drug development.

Mathematical modeling is more than just an alternative approach to understanding GPCR signaling and drug effects. Mathematical models quantify the GPCR system by offering a platform for numerical simulation of the interaction between receptors and ligands. In doing so, they can provide a quantitative description of both binding and function, as well as cooperativity factors between ligands. Existing mathematical models focus on the simulation of a single GPCR or its homomers, but scarcely address the issue of heteromerization. Therefore, a new model for GPCR heteromers is needed so as to quantitatively describe the influence of heteromerization on drug effects. Given that there are some features shared by GPCR monomers, homomers, and heteromers (such as allosteric interactions), it can be helpful to learn from previous mathematical models when constructing a new heteromer model.

There are a variety of mathematical models to formulate how functional effects change with drug concentration [11-13]. In regards to this work, the operational models of agonism and allosterism [14-17] are of particular interest. Previously, our group has significantly contributed to both the development of mathematical models for homomers [18-21] and the analysis of operational models of agonism [22-24]. Here, taking advantage of previous models, especially the operational models of receptor activation including constitutive receptor activity formulated by David Hall [16,17], we develop a model for receptor heteromerization. The translation of the

operational parameters for allosteric cooperativity in a monomer to the crosstalk between protomers in a heterodimer brings the utility of the pharmacological concepts present in the operational models to the heterodimer model and facilitates the application of widely used modeling and fitting procedures.

The model we present here is restricted to receptor heterodimers. Thus, it perfectly fits mGlu class C GPCRs, which are known to form strict dimers [25,26]. For class A GPCRs, a higher level of complexity is found in a number of cases and different equilibria between oligomers of varying degree of oligomerization have been described [2]. We view the present work as a first step in the mathematical modeling of receptor heteromerization by analyzing the simplest situation: a strict receptor heterodimer. This analysis sets up the basis for future work, which will include higher order hetero-oligomerization.

An operational model for the crosstalk between protomers in a receptor heterodimer

Figure 1 visualizes how the allosteric interactions between the orthosteric and allosteric sites in a monomer can qualitatively correspond to those between the orthosteric sites in a heterodimer. The quantitative formulation of this suggested correspondence is outlined in Figure 2.

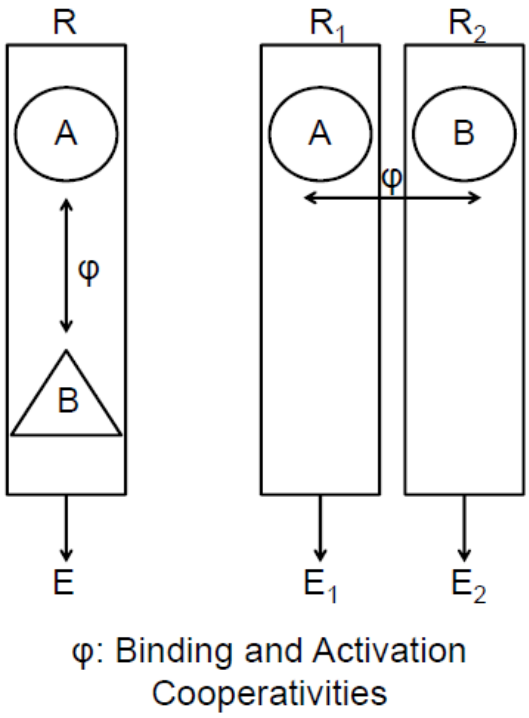
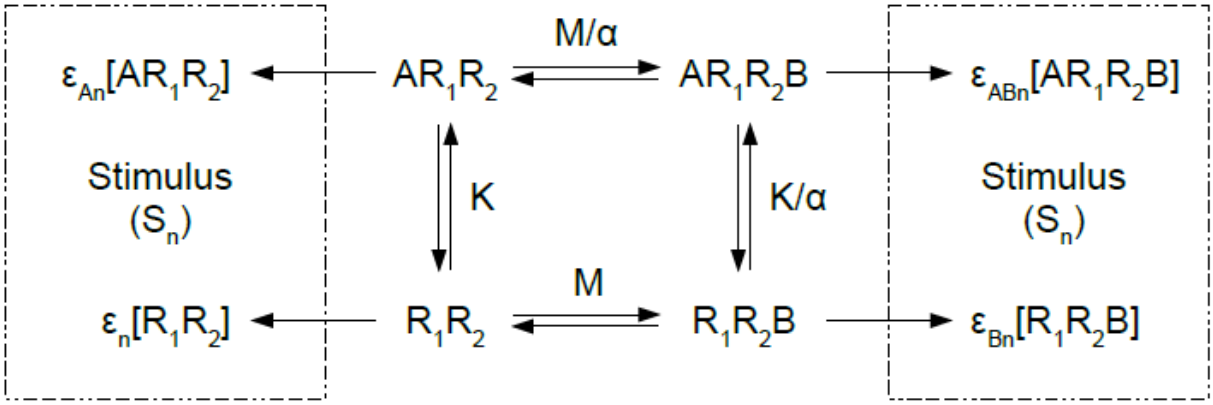


Figure 1. Qualitative scheme showing the correspondence of binding and activation cooperativities (ϕ) between the orthosteric and allosteric sites in a monomer, R (left), and the orthosteric sites of the protomers in a heterodimer, R_1R_2 (right). In the monomer, ligands A and B modulate each other to yield a receptor effect, E. In the heteromer, two receptor effects (E_1 and E_2) associated to their respective protomers (R_1 and R_2) are separately produced.

Figure 2 presents a mathematical model for a heterodimer consisting of two different receptors R_1 and R_2. R_1 and R_2 separately mediate pathway 1 and pathway 2. A and B are the ligands for R_1 and R_2, respectively. Owing to the conceptual correspondence between allosteric interactions in a monomer and in a heteromer shown in Figure 1, the rationale used in [17,22] for

the development of an operational model of allosterism for a receptor with constitutive activity was used here for a heterodimer.



$$f_n = \frac{E_n}{E_{mn}} = \frac{S_n}{K_{En} + S_n}$$

$$S_n = \varepsilon_n \times [R_1 R_2] + \varepsilon_{An} \times [AR_1 R_2] + \varepsilon_{Bn} \times [R_1 R_2 B] + \varepsilon_{ABn} \times [AR_1 R_2 B]$$

Figure 2. An operational model for a receptor heterodimer. Four receptor species (free, singly-bound and doubly-bound receptor molecules) are at equilibrium. Each of these receptor species has the ability to contribute to pathway stimulation. A rectangular hyperbolic function for the fractional effect on pathway n ($f_n=E_n/E_{mn}$) is proposed, with E_n being the effect; E_{mn}, the maximum possible effect in the system; S_n, the stimulus; and K_{En}, the value of S_n for $E_n/E_{mn}=1/2$. See main text and Table 1 for further description of the parameters.

The parameters present in Figure 2 have the following definitions (Table 1): K and M are the dissociation constants for the binding of ligands A and B to protomers R_1 and R_2, respectively. α

represents the binding cooperativity between the two ligands in their corresponding receptors. n (1 or 2) is used to distinguish between the two pathways. f_n denotes the fractional response of pathway n. S_n is the stimulus for functional response of pathway n. A rectangular hyperbolic function transduces stimulus S_n into fractional response f_n. E_n represents the produced effect for pathway n and E_{mn} denotes the maximum possible effect of the system for pathway n. K_{En} is the value of S_n for half of E_{mn}, so it measures the efficiency of transducing stimulus into fractional response. There is a difference in the number of parameters between the present model and its parent formulation [17,22], originally designed to account for the allosteric interactions between two ligands in a single receptor (Figure 1, left). The term ε_n, with n equal to 1 (for pathway 1) or 2 (for pathway 2), is used here to define the ability of the free R_1R_2 receptor to generate the functional response. In the original formulation a value of 1 was assumed for ε. Here, because of the possibility of two pathways, the term ε_n needs to be included.

Table 1. Definitions of parameters included in Equation 1

K	Dissociation equilibrium constant for the binding of ligand A
M	Dissociation equilibrium constant for the binding of ligand B
α	Binding cooperativity between ligands A and B
ε_n	Ability of R_1R_2 molecular entity to activate pathway n
ε_{An}	Ability of AR_1R_2 molecular entity to activate pathway n

ε_{Bn}	Ability of R_1R_2B molecular entity to activate pathway n
δ_n	A measure of the functional interactions between ligands A and B for pathway n. δ_n is included in the definition of $\varepsilon_{ABn} = \varepsilon_{An}\varepsilon_{Bn}\delta_n$, where ε_{ABn} is the ability of AR_1R_2B molecular entity to activate pathway n
χ_n	$\chi_n=[R_1R_2]_T/K_{En}$, with K_{En} defined in $f_n = \frac{E_n}{E_{mn}} = \frac{S_n}{K_{En}+S_n}$, where E_n is the effect, E_{mn}, the maximum possible effect and S_n the stimulus for pathway n

For pathway n, $\varepsilon_{An}/\varepsilon_n$ defines the intrinsic efficacy of ligand A; $\varepsilon_{Bn}/\varepsilon_n$ defines the intrinsic efficacy of ligand B; and $\delta_n*\varepsilon_n$ defines the activation cooperativity between ligands A and B. Intrinsic efficacies and cooperativities are considered positive, null and negative when they are greater than, equal to and lower than 1, respectively.

$K = [A] * [R_1R_2] / [AR_1R_2]$

$M = [B] * [R_1R_2] / [R_1R_2B]$

$M/\alpha = [B] * [AR_1R_2] / [AR_1R_2B]$

$K/\alpha = [A] * [R_1R_2B] / [AR_1R_2B]$

ε_{ABn}, ε_{An}, and ε_{Bn} denote the ability of AR_1R_2B, AR_1R_2, and R_1R_2B to activate pathway n, respectively.

Because ε_n is not necessarily 1, the intrinsic efficacies of A-B combination, A, and B for pathway n are the ratios $\varepsilon_{ABn}/\varepsilon_n$, $\varepsilon_{An}/\varepsilon_n$, and $\varepsilon_{Bn}/\varepsilon_n$, respectively.

δ_n is introduced to measure the functional interaction: $\varepsilon_{ABn} = \varepsilon_{An} * \varepsilon_{Bn} * \delta_n$

The activation cooperativity between A and B in the AR_1R_2B complex for pathway n is $(\varepsilon_{ABn}/\varepsilon_n) / ((\varepsilon_{An}/\varepsilon_n) * (\varepsilon_{Bn}/\varepsilon_n)) = \delta_n * \varepsilon_n$.

χ_n is a parameter used to account for the basal response of pathway n.

$\chi_n = [R_1R_2]_T / K_{En}$, with $[R_1R_2]_T = [R_1R_2] + [AR_1R_2] + [R_1R_2B] + [AR_1R_2B]$

Values greater than, equal to, and lower than one for the subsequent parameters or parameter combinations mean the following: (i) For α, positive, null, and negative binding cooperativities, respectively. (ii) For $\varepsilon_{An}/\varepsilon_n$ and $\varepsilon_{Bn}/\varepsilon_n$, positive, null, and negative intrinsic efficacies of ligands A and B, respectively. (iii) For $\delta_n * \varepsilon_n$, positive, null, and negative activation cooperativities, respectively.

Based on the aforementioned relationships, Equation 1 for the fractional effect f_n can be obtained (see Appendix 4b in [22] for the rationale followed).

$$f_n = \frac{\chi_n([A][B]\alpha\delta_n\varepsilon_{An}\varepsilon_{Bn}+[A]M\varepsilon_{An}+[B]K\varepsilon_{Bn}+KM\varepsilon_n)}{[A][B]\alpha(\chi_n\delta_n\varepsilon_{An}\varepsilon_{Bn}+1)+[A]M(\chi_n\varepsilon_{An}+1)+[B]K(\chi_n\varepsilon_{Bn}+1)+KM(\chi_n\varepsilon_n+1)} \quad (1)$$

The basal fractional response without ligands is $f_n = \frac{\varepsilon_n\chi_n}{\varepsilon_n\chi_n+1}$. It is worth noting that in basal conditions, the total receptor concentration is equal to the free receptor concentration ($[R_1R_2]_T = [R_1R_2]$).

By changing the values of the parameters in Equation 1, the model can be used to simulate different situations and test various hypotheses about the impact of ligand-receptor interactions on the signaling system. An example follows.

Ligands may substantially alter the relative activity of a receptor heterodimer: changing dominance

In order to explore how two ligands regulate the functional responses elicited by R_1 or R_2, we used two imaginary ligands with the parameter values set in Figure 3. A value of 10 for α shows that there is a positive binding cooperativity between the two ligands. Values for $\delta_1 * \varepsilon_1$ of 5 and $\delta_2 * \varepsilon_2$ of 0.5 indicate the positive activation cooperativity between A and B for pathway 1 and the negative activation cooperativity between A and B for pathway 2. The comparison between ε_1 and ε_2 shows the dominance of R_1 and pathway 1 over R_2 and pathway 2 when no ligands are bound to the heterodimer.

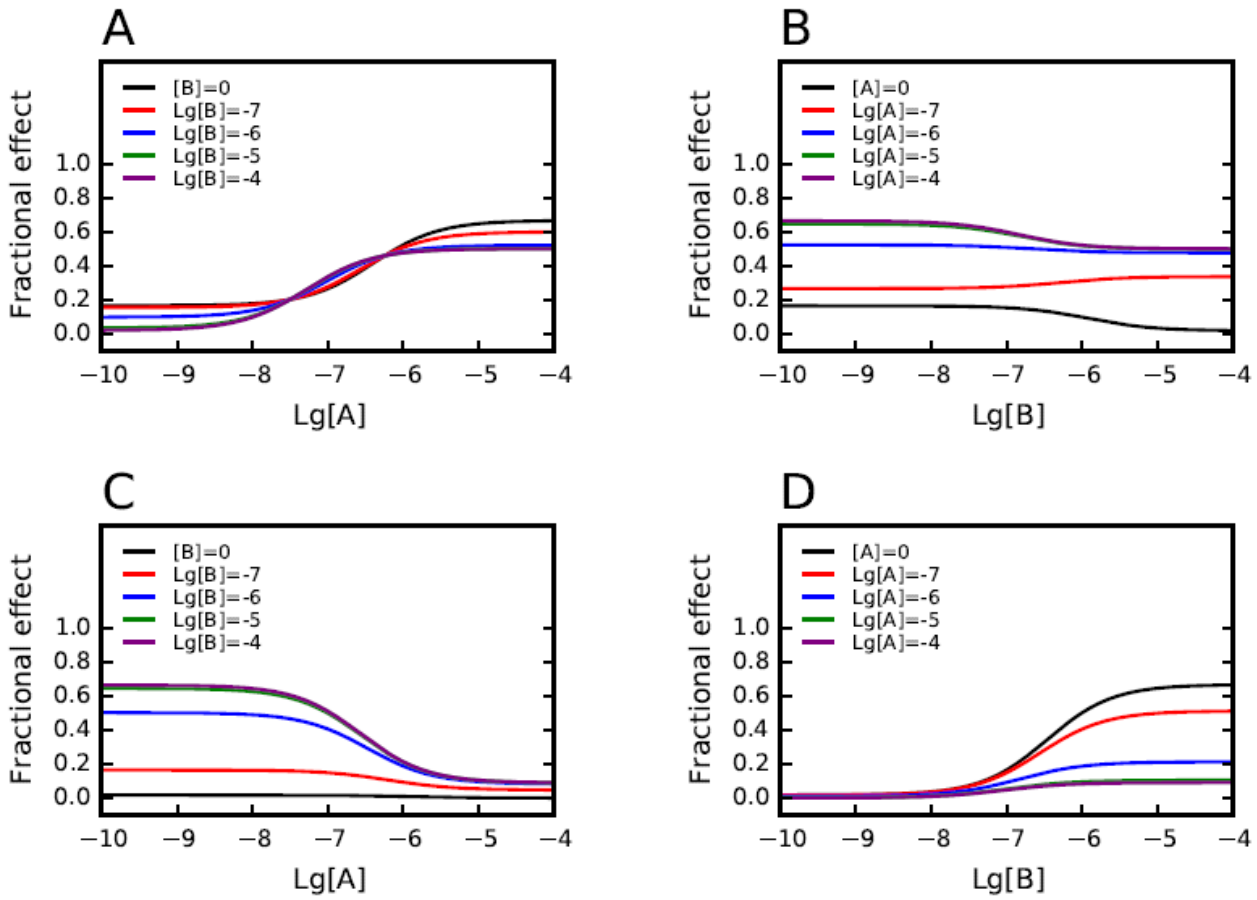


Figure 3. (A) and (B): Fractional effect for R_1-mediated pathway varies with the concentrations of ligands A and B. (C) and (D): Fractional effect for R_2-mediated pathway varies with the concentrations of ligands A and B.

Parameter setting:

Binding parameters			Functional parameters: Pathway 1					Functional parameters: Pathway 2				
K	M	α	χ_1	δ_1	ε_1	ε_{A1}	ε_{B1}	χ_2	δ_2	ε_2	ε_{A2}	ε_{B2}
10^{-6}	10^{-6}	10	0.2	5	1	10	0.1	0.2	5	0.1	0.01	10

The state of dominance can be modulated by the ligands. According to the values of ε_1, ε_{A1}, and ε_{B1}, ligand A promotes the activation of pathway 1, while B inhibits it. On the contrary, the values of ε_2, ε_{A2}, and ε_{B2} reveal that whereas A hampers the activation of pathway 2, B facilitates it. Here, ligand B changes the dominant protomer, increasing the efficacy of R_2 with respect to R_1.

Using these parameter values, the fractional effects of two pathways can be obtained (Figure 3). Figure 3 displays how two different ligands with positive binding cooperativity and positive or negative activation cooperativity interact to affect the functional responses of pathways 1 and 2. From Figures 3A and 3B it is shown that ligand A always increases the functional response of pathway 1, but the influence of ligand B on the pathway depends on the concentration of A. Figures 3C and 3D show that ligand A constantly decreases the functional response of pathway 2, while ligand B has the opposite effect.

It is worth noting that the functional responses obtained in Figure 3 result from the particular set of parameters we have chosen. A different set of parameters would lead to different plots and many combinations of positive and negative intrinsic efficacies and cooperativities are possible. In this regard, we have chosen a positive binding cooperativity (α=10). However, for many GPCR dimers, a negative cooperativity for ligand binding has been reported [$_2$]. Decreasing the binding cooperativity has as main effects a reduction in the apparent affinity and potency of the compounds, which results in a displacement of the concentration-effect (E/[A]) curves to the right. It is also interesting to comment on the intrinsic efficacy values. As

an example, for pathway 2, we have $\varepsilon_{B2}/\varepsilon_2$=10/0.1=100 as the intrinsic efficacy value of ligand B. As ε_2=0.1, lowering ε_{B2} to 1 still results in an agonist ligand: $\varepsilon_{B2}/\varepsilon_2$=1/0.1=10. However, making ε_{B2}=0.1 would make ligand B a neutral antagonist. Finally, making ε_{B2}<0.1 would convert ligand B into an inverse agonist.

Concluding remarks

Proteins usually act together to regulate biological activities [27]. As proteins, GPCRs often form homomers and heteromers under physiological conditions, providing new opportunities for drug design based on allosteric interactions between different receptors.

Life is a quantitatively observable process in principle. In this era of quantitative biology, mathematical modeling can greatly enhance our understanding of life and its processes. Efforts in this direction include not only genome-scale constructions but also the models of particular pathways or ligand-receptor systems. GPCR monomers and homomers have been modeled, but the heteromers have been scarcely addressed. In this study, we propose a mathematical model for the allosteric interactions within a GPCR heterodimer. This model quantifies the functional effects of ligands with different properties on GPCR-mediated signaling pathways. Our model for a receptor heterodimer is based on a previous operational model for the allosteric effects

between two binding sites in a single monomeric receptor. The resulting E/[A] equation can be used for simulation and fitting purposes. For the former case, an example has been given. For the latter case, it must be said that it is known that operational models cannot fit a single E/[A] curve [28]. Because of this, some conditions, such as those included in the irreversible inactivation method [29], need to be established. These conditions keep some of the parameters constant, thus enabling fitting [28]. With proper experimental data, the applicability of the present model of a receptor heterodimer for fitting purposes can be tested.

An example is given to illustrate the dominance of one protomer over the other within the heterodimer. In this example, the dominance appears because the two receptors differ in the ability to activate signaling pathways. However, the dominance may also be caused by their discrepancy in ligand binding. Provided that one receptor prevents another from binding to the agonist, the former becomes the dominant protomer by inhibiting the activation of the latter. This phenomenon occurs in the heterodimer consisting of serotonin 2C and one of serotonin 2A and 2B [30]. This level of dominance can also be described using our model by adjusting the dissociation constants of ligand-receptor pairs.

Overall, our receptor heterodimer model can be employed to quantify the ligand-receptor system. With more GPCR heterodimers being discovered and their functionality assessed, our model can only be of greater utility. Finally, functional data of complex receptor composition might be attributed to heterodimers if their related experimental results agree with the present model.

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4.2. The second work

An operational model for GPCR homodimers and its application in the analysis of biased signaling

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Keywords: GPCR; homodimer; operational model; constitutive activity; cooperativity; signaling pathway; biased agonism

Teaser: An operational model for GPCR homodimers including constitutive receptor activity is presented. The model explains typical pharmacological profiles associated with cooperativity in the framework of the operational model of agonism, thus taking advantage of the widespread use of this type of modeling. The operational homodimer model can also explain the biased signaling dependent on ligand concentration.

Abstract

G protein-coupled receptors are among the most important protein superfamilies as drug targets in drug discovery programs. Their interactions with ligands are influenced by their homomerization. In this study, we propose an operational model for receptor homodimers, which includes constitutive receptor activity. Distinct functional response curves can be obtained from this model, which can satisfactorily depict typical complex experimental data as biphasic and bell-shaped curves. Operational parameters in the model may provide mechanistic explanations for observed functional complexity associated with the cooperativity and intrinsic efficacy of ligands. Because the herein presented model is derived within the conceptual framework of operational models, it takes advantage of the body of knowledge coming from the widespread use of this type of modeling. The operational homodimer model can also explain the biased signaling dependent on ligand concentration. In conclusion, this operational homodimer model has a wide range of applications in pharmacological research.

Introduction

G protein-coupled receptors (GPCRs) are integral cell membrane proteins responsible for many biological processes. Extracellular agonists bind to these receptors and influence their conformations, thus altering the propensity of receptors to bind to G proteins, β-arrestins, or other transducer proteins and promoting signal transduction within the cell. In this way, GPCRs respond to extracellular stimuli and help to direct the information flow from outside to inside the cell [1]. The analysis of drug-GPCR interactions is fundamental in both academia and pharmaceutical industry. Because of the involvement of GPCRs in many physiological processes, their malfunctioning is the cause of many diseases. As a consequence, research on GPCRs is central to drug discovery programs. Of note, as of November 2017, GPCRs are the primary targets of approximately 35% of approved drugs in the United States or European Union [2].

It is widely accepted that GPCRs often form oligomers which may be physiologically relevant [3]. The formation of oligomers affects the binding of extracellular ligands to GPCRs and enables the ligands to produce a wider and more complex range of functional responses [4].

Homodimerization is the simplest case of receptor oligomerization. The allosteric interactions between the bound ligands in the respective protomers may lead to cooperativity effects which can affect the binding and the function of the receptor [5,6]. Ligand binding cooperativity in a receptor dimer context has been the subject of modeling approaches [7,8]. Durroux considered the possibility of two different receptor dimer states, one in which receptor protomers are able

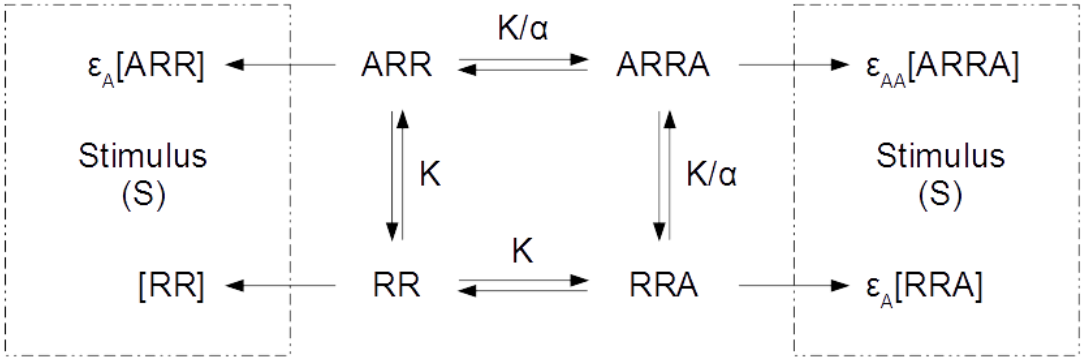
to crosstalk and the other in which they are not [7]. Casadó et al. comprehensively compared procedures to fit binding data from both saturation isotherms and competition assays within a receptor dimer model with the traditional way of fitting data [8]. The issue of fitting of binding data was reviewed by Giraldo [9], who analyzed various empirical and mechanistic models. Moreover, the functional response induced by ligands can also be assessed by mathematical models. There have been some mathematical models for signaling mediated by GPCR homodimers [10-12]. However, no operational model has been proposed for this purpose. The operational model of agonism was presented in 1983, but it considered the receptor as a monomer [13]. This model describes the receptor signaling as a two-step process, one for ligand binding and the other for transducing the ligand-receptor complex into the functional response. This model has been widely used to analyze the functional effects of ligands and, remarkably, the issue of biased agonism [14,15]. Nevertheless, it cannot explain the behavior of inverse agonists. This is because the constitutive activity of the receptor is not integrated into the model. Subsequently, there have been extensions of the operational model by incorporating constitutive receptor activity, but they are still intended for the monomer [16,17]. Therefore, here we aim to propose an operational model for GPCR homodimers. This new model considers constitutive receptor activity and thus is intended to describe the function of inverse agonists.

One GPCR may perform its functions through a variety of pathways. The same ligand is likely to generate different effects on different pathways. When distinct ligands bind to the receptor, it is possible that different sets of downstream pathways will be affected. Biased signaling occurs

for many ligands binding to GPCRs in the human body and has great significance in biological functions [1]. Therefore, we have applied the herein developed operational homodimer model to the dissection of biased signaling by considering different pathways which are associated with one receptor homodimer. Remarkably, the model assists in the understanding of concentration-dependent effects of a ligand on different pathways.

An operational model for GPCR homodimers

Figure 1 illustrates an operational model for a GPCR homodimer consisting of two protomers R. The combination of the two protomers in the homodimer mediates the functional response. A is a ligand for each of the protomers. ARR and RRA are equivalent.



$$f=\frac{E}{E_m}=\frac{S}{K_E+S}$$

$$S=[RR]+\varepsilon_A\times[ARR]+\varepsilon_A\times[RRA]+\varepsilon_{AA}\times[ARRA]$$

Figure 1. An operational homodimer model.

$K = [A] * [RR] / [ARR]$

$K = [A] * [RR] / [RRA]$

$K/\alpha = [A] * [ARR] / [ARRA]$

$K/\alpha = [A] * [RRA] / [ARRA]$

K is the equilibrium dissociation constant for the singly-bound receptor dimer. α is the constant for binding cooperativity. f denotes the fractional functional response ($f=E/E_m$), with E and E_m being the absolute functional response and the maximum possible functional response of the system, respectively. S is the stimulus for the functional response. K_E is the value of S for half of E_m and thus represents the efficiency of transducing stimulus into fractional response. ε_{AA} and ε_A are the intrinsic efficacies of A-A combination and A, respectively.

The parameters in this model have the following definitions. K is the equilibrium dissociation constant for the binding of ligand A to the free homodimer. α represents the binding cooperativity between the two ligands. For α, values less than, equal to, and greater than 1 mean negative, neutral, and positive binding cooperativities, respectively. E denotes the absolute functional response and E_m is the maximum possible functional response of the system. f denotes the fractional functional response ($f = E/E_m$). S is the stimulus for the functional response. A rectangular hyperbolic function converts stimulus S into fractional response f. K_E is the value of S for half of E_m and thus represents the efficiency of transducing

stimulus into fractional response. ε_{AA} and ε_A are the intrinsic efficacies of A-A combination and A, respectively. δ is introduced to measure the functional interaction: $\varepsilon_{AA} = \varepsilon_A * \varepsilon_A * \delta$; meaning that δ is the activation cooperativity between A and A in the ARRA complex. χ is a parameter used to account for the basal fractional response. $\chi = [RR]_T / K_E$, with $[RR]_T$ = [RR] + [ARR] + [RRA] + [ARRA]. Given that the constitutive activity of the receptor is considered here, this model can be used to analyze the functional effects of inverse agonists. This feature distinguishes our model from many other models.

Based on above relationships, Equation 1 for the fractional response f of the homodimer model depicted in Figure 1 can be obtained.

$$f = \frac{\chi\left(K^2 + 2K\varepsilon_A[A] + \alpha\delta{\varepsilon_A}^2[A]^2\right)}{K^2(\chi+1) + 2K(\chi\varepsilon_A+1)[A] + \alpha\left(\chi\delta{\varepsilon_A}^2+1\right)[A]^2} \qquad (1)$$

The asymptotic response when $[A]\rightarrow\infty$ (f_∞) is calculated as

$$f_\infty = \lim_{[A]\rightarrow\infty} f = \frac{\chi\delta{\varepsilon_A}^2}{\chi\delta{\varepsilon_A}^2+1} \qquad (2)$$

The basal fractional response of the system (f_{basal} = f for [A]=0) is

$$f_{basal} = \frac{\chi}{\chi+1} \qquad (3)$$

As displayed by Equation 3, f_{basal} is determined by χ and rises as χ increases. Therefore, χ is associated with the intrinsic ability of a signaling system without ligands to generate the functional response. Equation 2 shows that f_∞ is positively correlated with χ, δ, and ε_A. It is very interesting to note that both f_{basal} and f_∞ are influenced by χ. This means that f_{basal} and f_∞ are interrelated and highlights the importance of the measurement of basal response in the analysis of ligand intrinsic efficacy.

Different parameter values lead to functional response curves with different shapes

According to the model for the receptor homodimer (Figure 1 and Equation 1), a variety of curves with differing shapes can be obtained when different values are assigned to the parameters. In order to better understand how the parameter values affect the functional response curves, we conducted some simulations using the following values and special attention was paid to the comparison between ε_{AA}, ε_A, and 1.

The nine curves in Figure 2 share the values for χ, K, and α: χ = 0.5, K = 10^{-8}, and α = 0.001, so there is a negative binding cooperativity between the two ligands binding to the homodimer. The following nine curves have distinct values of ε_{AA} and ε_A: (1) ε_{AA}=100, ε_A=10; (2) ε_{AA}=10, ε_A=10; (3) ε_{AA}=1, ε_A=10; (4) ε_{AA}=10, ε_A=1; (5) ε_{AA}=1, ε_A=1; (6) ε_{AA}=0.4, ε_A=1; (7) ε_{AA}=1, ε_A=0.4; (8)

ε_{AA}=0.4, ε_A=0.4; (9) ε_{AA}=0.1, ε_A=0.4. The basal fractional response depends exclusively on the χ parameter, which reflects the constitutive receptor activity. Because χ is constant in all the simulations depicted in Figure 2, the basal fractional response is also constant, and equal to 0.33.

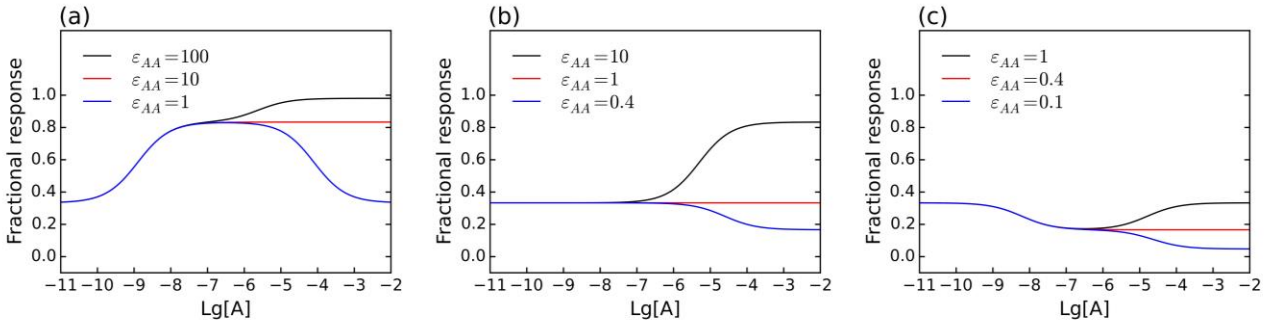


Figure 2. Simulated functional response curves obtained through Equation 1. χ = 0.5, K = 10^{-8}, and α = 0.001. Various conditions of intrinsic efficacy are considered: (a) ε_A = 10; (b) ε_A = 1; (c) ε_A = 0.4.

Figure 2 shows the curves with distinct shapes. (1) When $\varepsilon_{AA} > \varepsilon_A > 1$, the curve is biphasic; (2) When $\varepsilon_{AA} = \varepsilon_A > 1$, the curve is monophasic; (3) When $\varepsilon_{AA} < \varepsilon_A > 1$, the curve displays an inverted U-shape pattern; (4) When $\varepsilon_{AA} > \varepsilon_A = 1$, the curve is monophasic; (5) When $\varepsilon_{AA} = \varepsilon_A = 1$, ligand A is a neutral antagonist, and the curve is a horizontal straight line; (6) When $\varepsilon_{AA} < \varepsilon_A = 1$, the curve is monophasic; (7) When $\varepsilon_{AA} > \varepsilon_A < 1$, the curve is U-shaped; (8) When $\varepsilon_{AA} = \varepsilon_A < 1$, the curve is monophasic; (9) When $\varepsilon_{AA} < \varepsilon_A < 1$, the curve is biphasic. These functional response curves illustrate how ligands influence receptor function. If an experimental result agrees with

one of these curves, this operational homodimer model can serve as a possible explanation for the observation.

Understanding intrinsic efficacy and cooperativity by comparing our model with the two-state dimer model

Ligand intrinsic efficacy and cooperativity are key concepts in mechanistic pharmacology. However, different mathematical models may address them from distinct perspectives. In order to gain a better understanding of intrinsic efficacy and cooperativity, we have made an attempt to compare the present operational homodimer model with the two-state dimer receptor model [10].

The two-state dimer receptor model (Figure 3) regards the homodimer as a global functional entity. This entity has one active state and one inactive state. The active state is responsible for the downstream functional response. The homodimer has two ligand binding sites. The ligand affects the proportion of active homodimers by means of conformation selection or induction. In this model, (R_2) and (R_2)* denote the inactive and active receptor homodimers, respectively. A represents the ligand binding to the homodimer. K is the equilibrium association constant between A and (R_2). L explains the constitutive activity of the homodimer. α and β denote the intrinsic efficacies of the first and the second ligands binding to the homodimer, respectively. As

a result, β/α is the activation cooperativity between the two ligands. When β/α = 1, the first ligand does not affect the function of the second ligand. In this case, μ is the binding cooperativity between the two ligands.

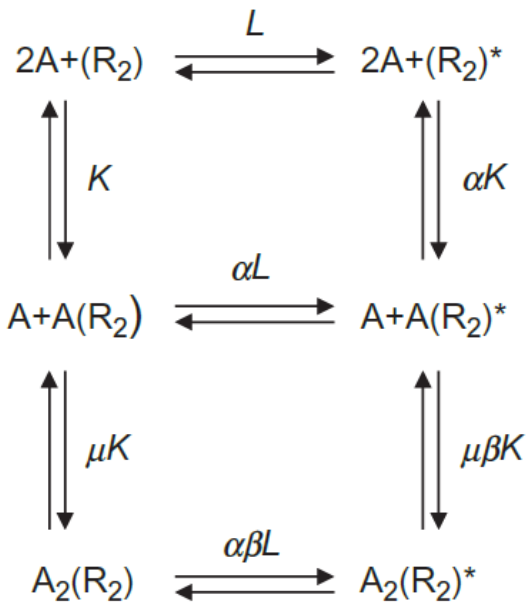


Figure 3. The two-state dimer receptor model [10].

Table 1 shows the parameter comparison between our model and the two-state dimer receptor model [10]. As displayed in the table, there is good agreement in the concepts of intrinsic efficacy and cooperativity between the two models. Interestingly, the two models have similar definitions of ligand intrinsic efficacy. Specifically, both models use the free homodimer as the reference to assess ligand function. In the operational homodimer model, the ability of the free

homodimer has a value of 1, so the relative ability of the first ligand to activate the pathway is ε_A, which is its intrinsic efficacy. In the two-state dimer model, the transformation constant is L for free homodimer and αL for singly occupied homodimer, so the intrinsic efficacy of the first ligand is α, the ratio of αL to L. It is necessary to use this reference when evaluating ligand intrinsic efficacy because the reference enables us to eliminate the impact of non-ligand factors. In this way, it is feasible to compare the intrinsic efficacies of ligands from different experiments.

Table 1. Correspondence between parameters in the operational homodimer model and the two-state dimer receptor model [10].

Meaning of the parameter	**Operational homodimer model**	**Two-state dimer receptor model**
Constant related to constitutive receptor activity	χ	L
Intrinsic efficacy of the first ligand	ε_A	α
Intrinsic efficacy of the second ligand	$\varepsilon_{AA}/\varepsilon_A = \varepsilon_A \delta$	β
Activation cooperativity	δ	β/α
Binding cooperativity	α	μ (when β/α = 1)

The operational homodimer model can be used for the analysis of biased signaling and concentration-dependent ligand bias

A ligand may differentially impact multiple cellular pathways by binding to the same receptor. Provided that the receptor exists in the form of homodimers, our operational homodimer model can help to explain the biased signaling of the ligand. The receptor interacts with different downstream proteins for different pathways, so the parameters in this model may have varying values for these pathways, and each pathway can be represented by a set of parameter values.

Ligand bias may be affected by ligand concentration. At lower concentrations, the ligand may prefer pathway A to pathway B. Nevertheless, the same ligand may preferentially activate pathway B at higher concentrations. This phenomenon can be explained by assuming the ligand stabilizes different receptor conformations at different concentrations. A plausible explanation is that the receptor can bind to more than one ligand and receptors with different numbers of bound ligands may differ in their conformational ensembles. The discovery of receptor homomerization supports this explanation. For example, a singly occupied homodimer may have different preferential conformations from a doubly occupied one. Ligand bias may vary with the number of ligands binding to the homodimer.

In an experimental study, Sun et al. [18] found that the signaling mediated by β_2-AR changed from G-protein-dependent to G-protein-independent with the increase in ligand concentration.

Biphasic dose-response curves were obtained [18]. In a previous paper, our laboratory explained this finding using the asymmetric/symmetric three-state dimer model [12]. We inferred that the asymmetric active state of the receptor dimer functions by G-protein-dependent pathway, whereas its symmetric active state acts on G-protein-independent pathway. This assumption led to functional response curves in agreement with the experimental results of Sun et al. [18]. The herein presented operational homodimer model can describe these results from another perspective. It can be assumed that the singly occupied dimer and the doubly occupied dimer favor G-protein-dependent pathway and G-protein-independent pathway, respectively. Under this presumption, our model provides functional response curves (Figure 4) similar to those given by Rovira et al. [12]. Figure 4 illustrates how two different pathways are influenced by the same ligand with varying concentrations. At low concentrations, the ligand prefers the G-protein-dependent pathway to the G-protein-independent pathway. However, the preference is inverted when ligand concentration exceeds a threshold. This concentration-dependent ligand bias is in accordance with the observation for the β_2 adrenergic receptor made by Sun et al. [18].

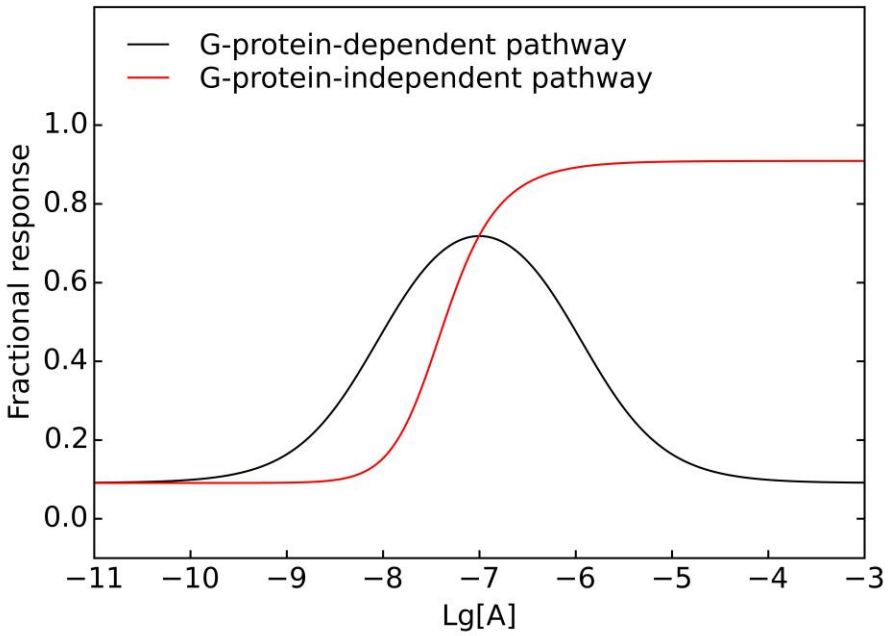


Figure 4. Ligand bias changes with ligand concentration. The two curves share three parameter values: K = 10^{-7}, χ = 0.1, and α = 1. G-protein-dependent pathway: ε_{AA} = 1, and ε_{A} = 50. G-protein-independent pathway: ε_{AA} = 100, and ε_{A} = 1. The setting of ε_{AA} and ε_{A} for the two pathways is based on the assumption that singly-bound dimers and doubly-bound dimers favor G-protein-dependent pathway and G-protein-independent pathway, respectively. As ligand concentration increases, the initially formed singly-bound dimers convert into doubly-bound dimers. Accordingly, with the intrinsic efficacy parameters used, the G-protein-dependent signaling curve is of an inverted U-shape, whereas the G-protein-independent signaling increases monotonically. The combination of the two curves in the figure provides a mechanistic interpretation for the biphasic dose–response curve reported by Sun et al. [18].

Concluding remarks

Receptor oligomerization, particularly receptor homodimerization, provides complexity and flexibility for the signal transduction processes mediated by GPCRs. The formation of receptor homodimers influences ligand-GPCR interactions and consequently GPCR function. For instance, there may be binding and activation cooperativities between the first and the second bound ligands. The impact of the cooperativities on the effects of the ligand can be quantified by mathematical models, such as the present operational homodimer model and the two-state dimer receptor model. The herein presented operational homodimer model is an extension of previous operational models including constitutive receptor activity [16,17], which, in turn, are extensions of the seminal operational model of agonism [13]. All of the previous models were defined for monomeric receptors. Operational models have proved instrumental for the analysis of the functional response of pharmacological receptors. The model presented herein is intended particularly for those cases where receptor dimerization is suspected as the explanation for biphasic or bell-shaped curves.

Given that the same ligand may influence distinct pathways mediated by one receptor, it is necessary to take biased signaling into account in the analysis of functional effects of the ligand. Since the conformations of singly occupied receptors may be different from those of doubly occupied receptors and these conformations may be associated with different signaling pathways, the preference of the ligand for some pathways may vary with its concentration. The operational homodimer model can satisfactorily depict this concentration-dependent ligand bias. We consider that a signaling pathway is determined by the values of the set of parameters in the model (Equation 1). Thus, a particular set of parameter values represents a particular

pathway. In the example we have used for concentration-dependent ligand bias, we applied two sets of parameter values, so just two pathways were analyzed. If an additional set of parameter values is included, a third signaling pathway can be examined. In this way, the model can compare multiple pathways if their parameter values are known. Therefore, it may be concluded that the model provides a suitable platform for the analysis of biased signaling in a receptor homodimer context.

Appendix

Development of Equation 1

The fractional functional response f is defined as

$$f = \frac{E}{E_m} = \frac{S}{K_E + S} \qquad \text{(A1)}$$

with E and E_m being the absolute functional response and the maximum possible functional response of the system, respectively. S is the stimulus for the functional response. K_E is the value of S for half of E_m.

The stimulus S results from the sum of stimuli of the components of the system

$$S = S_{RR} + S_{ARR} + S_{RRA} + S_{ARRA} \qquad \text{(A2)}$$

$$S_{RR} = [RR]$$

$$S_{ARR} = \varepsilon_A[ARR]$$

$$S_{RRA} = \varepsilon_A[RRA]$$

$$S_{ARRA} = \varepsilon_{AA}[ARRA]$$

With ε_A and ε_{AA} being the intrinsic efficacies of A and A-A combination, respectively.

The relative populations of ligand and receptor species are regulated by the dissociation equilibrium constants

$$K = \frac{[A][RR]}{[ARR]} = \frac{[A][RR]}{[RRA]} \qquad \text{(A3)}$$

$$\frac{K}{\alpha} = \frac{[A][ARR]}{[ARRA]} = \frac{[A][RRA]}{[ARRA]} \qquad \text{(A4)}$$

With α being the binding cooperativity.

We replace the stimulus S in Equation A1 with its definition given in Equation A2 and subsequently put all the receptor species in terms of [RR]. Equation A5 is obtained.

$$f = \frac{\frac{[RR]}{[RR_T]}\left(1 + \frac{2\varepsilon_A[A]}{K} + \frac{\varepsilon_{AA}\alpha[A]^2}{K^2}\right)}{\frac{K_E}{[RR_T]} + \frac{[RR]}{[RR_T]}\left(1 + \frac{2\varepsilon_A[A]}{K} + \frac{\varepsilon_{AA}\alpha[A]^2}{K^2}\right)} \qquad \text{(A5)}$$

With $[RR_T] = [RR] + [ARR] + [RRA] + [ARRA]$

We define $\chi = \frac{[RR_T]}{K_E}$ and $\varepsilon_{AA} = \varepsilon_A{}^2\delta$, and take into account that $\frac{[RR]}{[RR_T]} = \frac{1}{1+\frac{2[A]}{K}+\frac{\alpha[A]^2}{K^2}}$

After some algebra, Equation A5 is transformed into Equation A6, which is the same as Equation 1 in the main text.

$$f = \frac{\chi\left(K^2 + 2K\varepsilon_A[A] + \alpha\delta\varepsilon_A{}^2[A]^2\right)}{K^2(\chi+1) + 2K(\chi\varepsilon_A+1)[A] + \alpha\left(\chi\delta\varepsilon_A{}^2+1\right)[A]^2} \qquad \text{(A6)}$$

Acknowledgements

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4.3. The third work

Biased signaling: covering the whole pharmacological space

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Abstract

Biased signaling is at the center of current pharmacological research in both academia and industry. The distinct and often opposed effects that a medication can exert by acting through different pathways mediated by the same receptor drive medicinal chemists to design molecules which interact with a specific pathway downstream of the receptor in order to improve therapeutic index. Efficient drug discovery is underpinned by rigorous quantitative pharmacology. Thus, reliable optimization of biased ligands requires robust scales of quantification applicable to the entire pharmacological ligand space: agonists, neutral antagonists and inverse agonists. To this aim, new scales for the quantification of system bias, ligand bias, and signaling bias are proposed here based on a novel unified framework of biased signaling. Importantly, the present article provides a new perspective for the origins of biased signaling.

On the quest of a pharmacological property to quantify GPCR biased signaling

G protein-coupled receptors (GPCRs) share a common structure of 7 transmembrane helices connected by extracellular and intracellular loops. These proteins are inserted in the cellular membrane and transmit the signals embodied in the structure of ligands from outside to inside

the cell [1]. There are a variety of endogenous ligands for GPCRs, including some hormones and neurotransmitters. GPCRs are allosteric machines by their nature [2, 3]. The endogenous agonists bind in the orthosteric site and then favor the binding of the G protein to the receptor at the intracellular site. There, the G protein is activated and its trimeric αβγ structure is broken into α and βγ subunits, which can transmit the signal to the effector and yield the physiological response [1].

GPCRs signal not only through the G protein pathway but also through other pathways, such as the β-arrestin pathway [4-6]. Engagement of this multiplicity of signaling pathways may have therapeutic implications [7-9]. For instance, typical opioid analgesics (such as morphine) directed to the μ-opioid receptor yield beneficial effects through the G protein pathway but unwanted side effects through the β-arrestin pathway [10]. Because of this, drug discovery efforts were focused on the G protein pathway mediated by this receptor [11], leading to the development of TRV130 [12] and PZM21 [13]. Similarly to the μ-opioid receptor, the implications of the dopamine D2 receptor biased signaling on schizophrenia [14, 15] and of the β-adrenergic receptors and of the angiotensin type I receptor for cardiovascular indications [7] have been reported.

Accurate quantification of drug effects is of primary importance [16]. This is of special relevance in biased signaling assays, because the rapid growing of the investigations on biased pharmacological agents and specific targeted therapeutics makes the potential appearance of systematic errors not negligible [17]. Thus, a scale is needed to quantify the biased agonism of ligands so that we can identify the quantitative preference of ligands for some signaling pathways. To this end, a relevant pharmacological property (X) should be identified. We can consider two possibilities: either this property is independent of receptor density or it is

proportional to receptor density (Figure 1). In the former case, comparisons can be made directly between pathways without use of a reference standard whilst, in the latter case, the property will require normalization to a reference compound. In both cases, the difference in the value of this property between two ligands must be unrelated to receptor density. We could use the potency of the ligand, expressed as the negative logarithm of EC_{50}, as a scale for the quantification of biased agonism. However, this scale is not appropriate as it provides a nonlinear plot in terms of the density of the receptor. The differences between two ligands depend on the level of receptor concentration and this happens regardless of the steepness of the **concentration-effect curves** (see Glossary) of the ligands [17]. Although the activity ratio (the ratio of the maximum response of an agonist to EC_{50}) works for rectangular hyperbola curves, that is, concentration-effect curves following the Hill equation with slope of 1, this scale is not appropriate either for steep curves or for flat curves [17]. Fortunately, the Black and Leff operational model of agonism provides an opportunity to solve this problem [17-19].

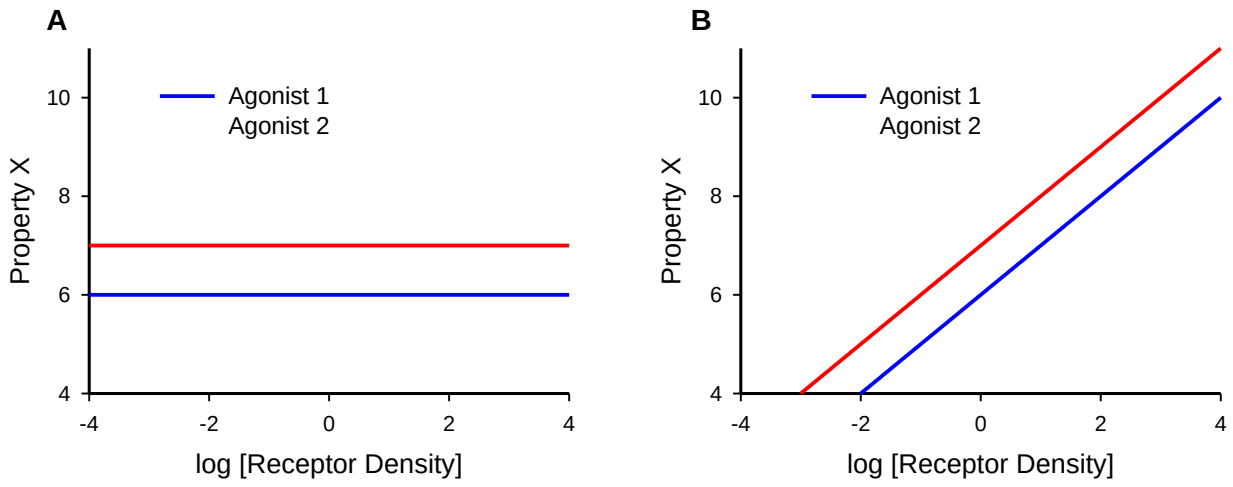

Figure 1. **A**. The pharmacological property X for a particular ligand-receptor complex does not change with receptor density, but there can be differences in X between ligands. **B**. The pharmacological property X for a particular ligand-receptor complex depends on receptor density but the difference in X value between two ligands is constant along receptor density.

The Black and Leff operational model of agonism (Box 1. Model 1) considers two steps for the generation of a pharmacological effect by a receptor [20]: the binding of the agonist to the receptor, which follows the law of mass action, and a logistic function for the transduction of binding into effect. In this equation, E_m is the maximum effect of the system and K_E is the concentration of agonist:receptor complex that induces a response equal to 0.5 E_m (Box 1. Equation 2).

The final concentration-effect equation (Box 1. Equation 3) contains the following parameters: E_m, K_A (the equilibrium dissociation constant), n (a parameter related to the slope of the curves), and τ. But, what is the meaning of τ? This parameter is the ratio of the total receptor concentration to K_E, the parameter determining the transduction of receptor binding into effect. Therefore, τ is proportional to receptor density. τ is the operational efficacy and determines the maximum response of a particular agonist and also affects the potency of the ligand. Accordingly, τ serves as a potential scale for biased agonism [21].

However, the concentration-effect curve is also greatly influenced by K_A in addition to τ. Consequently, the combination of the operational efficacy τ and the dissociation constant K_A has been proposed to quantify biased agonism [17]. Because τ is proportional to receptor

density, $\Delta\log(\tau/K_A)$ is independent of receptor density and would be an appropriate property to quantify the differences between the agonist of interest and the reference ligand (Figure 1B). The reference ligand is used here to cancel systematic differences in the sensitivity of the different signaling pathways to activated receptors. Finally, $\Delta\Delta\log(\tau/K_A)$ measures biased agonism between pathways for the particular agonist (Box 1).

The classical operational model of agonism [20] does not account for constitutive receptor activity and thus inverse agonists are excluded from the analysis of biased agonism. In a recent paper [22] we used a model which includes constitutive receptor activity in its definition (Box 1. Model 2). Both the free receptor and the ligand-bound receptor produce a stimulus (Box 1. Equation 8). ε is the intrinsic efficacy of the ligand. Depending on the value of ε, the ligands may be agonists, neutral antagonists, or inverse agonists. It was proposed [22] that $\log(\varepsilon/K_A)$ can work as a scale for biased agonism. Importantly, this scale does not depend on receptor density and thus it is not necessary to use a reference compound to cancel the system effect (Figure 1A).

A new conceptual framework for biased signaling

We have constructed a conceptual framework for biased signaling (Figure 2) which includes the constitutive receptor activity [23] and a new scale for biased agonism [22]. In this framework, we consider first the inherent bias of the signaling system (when the ligands are not present)

with respect to different pathways which we refer to as 'system bias' and then how the ligands introduce extra bias through their impact on the system.

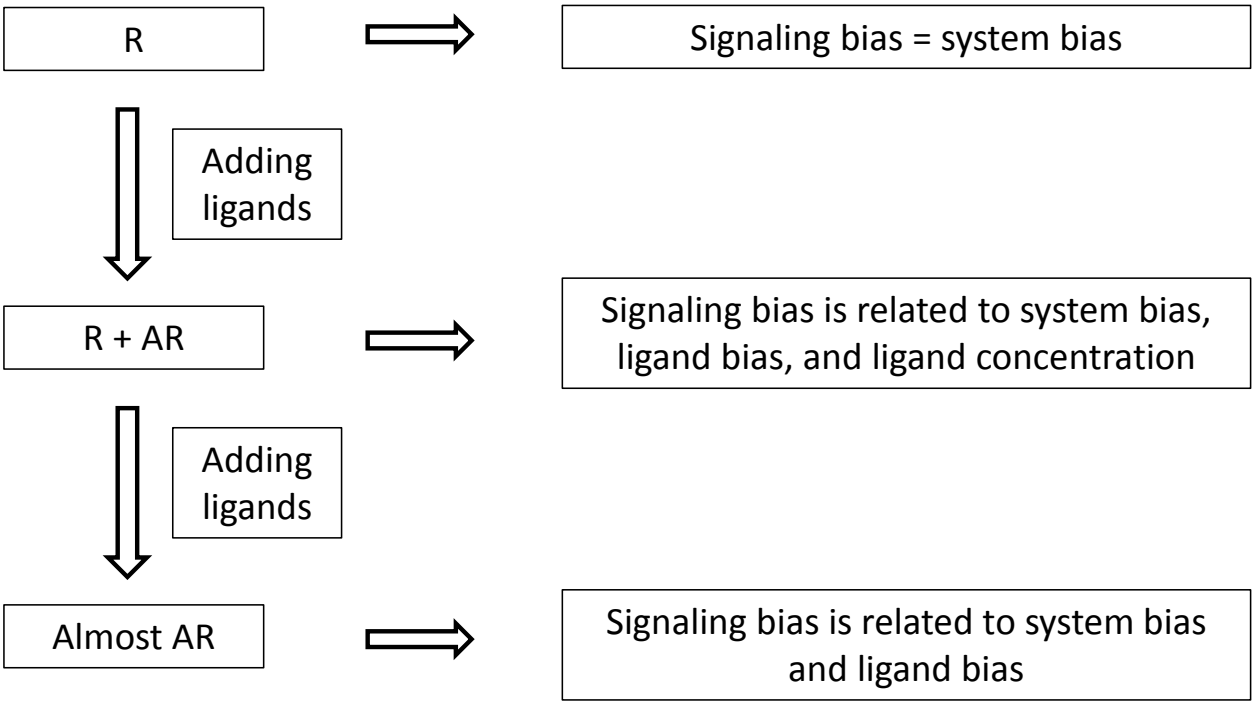

Figure 2. A conceptual framework for biased signaling. **Top of the chart:** in the absence of ligands, the free receptor may show a preference for a particular signaling pathway. Signaling bias is equal to system bias. **Middle of the chart:** after adding a certain amount of ligand, two receptor species (R and AR) are in equilibrium in the medium. Signaling bias depends on system bias, ligand bias and ligand concentration. **Bottom of the chart:** after adding a sufficient amount of ligand, almost all the receptor is in the form of AR complex. Signaling bias depends on system bias and ligand bias. Top, middle and bottom of the chart are consistent with, respectively, the left, middle and right parts of concentration-effect curves of constitutively active receptors on different pathways.

System bias

A **signaling system** (see Glossary) is composed of a receptor, various transducers, and various effectors participating in a particular biological assay. A pathway can be viewed as a sequence of biochemical events from the receptor to one functional endpoint in the signaling system. Several pathways can be examined in a signaling system if two or more functional endpoints are measured. A ligand has functional effects only after it is added into a signaling system. Because ligand effects are dependent on the composition of the signaling system, it is necessary to discuss the bias of the system before the analysis of ligand bias. Many signaling systems have detectable basal functional responses [24]. In other words, the pathway can be activated without ligand binding. If a signaling system has different levels of basal functional responses (also termed constitutive receptor activity) for two distinct pathways, this system can be thought to be biased towards the pathway with a higher basal response. Since it is not meaningful to directly compare the absolute functional responses of two different pathways, we need to standardize the functional responses and then compare their relative values. A natural reference for standardization is E_m and the relative values are then the fractional basal responses. E_m is defined as the maximal response of the signaling system for a specific pathway, so it represents the maximal potential of the system under consideration to generate a functional response for the pathway.

In this manner, the **system bias** (see Glossary) can be calculated as $\Delta\log(E_{basal}/E_m)$ or $\Delta\log(f_{basal})$, where E_{basal} denotes the basal response of this system for a particular pathway and f_{basal} the corresponding fractional functional response. The system bias is influenced by the components of this system. For example, the expression of the receptor and transducers can affect the basal

responses of different pathways. The system bias can be viewed as a combination of receptor bias, transducer bias, and effector bias. In the Slack & Hall operational model of agonism (with unit slope), f_{basal} is equal to $\chi/(1 + \chi)$, where χ is the ratio of $[R_T]$ (the total receptor concentration) to K_E (the total receptor concentration that results in a basal response of 0.5 x E_m). Therefore, system bias is determined by χ [23] (see also [25] for review).

Ligand bias

In the Slack & Hall operational model [23], the receptor R can be taken to represent a conformational ensemble of many conformations in equilibrium [22]. Different conformations may differentially interact with downstream signaling proteins. The impact of the ensemble on different pathways determines the constitutive receptor activity. The ligand L may change the influence of the receptor on downstream pathways by affecting the state of the ensemble [26]. In other words, the probability of occurrence (or, in other words, the stability) of each conformation in the ensemble when the receptor has a ligand bound may be different from that of unoccupied receptor. In this way, R and RL differ in influencing downstream pathways.

When analyzing the **ligand bias** (see Glossary) regarding different pathways, Kenakin et al. [17] used a reference ligand to remove system bias. However, the selection of the reference ligand is somewhat arbitrary. Although using the endogenous ligand seems to be a good choice, there are many receptors which there is more than one endogenous ligand. Under these conditions, it is an issue how to choose the reference ligand. Therefore, a better solution to this problem

could be to use a combination of the effects of the set of all potential ligands for a specific receptor as the reference for the function of this receptor. According to the conformational selection theory, receptors are always in equilibrium between various conformations. A ligand preferentially binds to some of them, making these conformations dominant over others. Therefore, a reasonable approximation for the average effect of the set of all potential ligands for a particular receptor is the conformational ensemble of the free receptor. It follows that the free receptor is a good surrogate of the potential ligand set and can serve as the reference against which ligand bias is assessed. By evaluating the difference between R and RL in their abilities to activate different pathways, we can know the intrinsic efficacies of the ligand for these pathways. The ligand may be an agonist, a neutral antagonist, or an inverse agonist according to its intrinsic efficacy.

As ligand effect is influenced by both intrinsic efficacy and affinity, we propose the concept of "ligand power" (pow) to measure the impact of the ligand on a pathway. Taking the Slack & Hall operational model [23] as a framework, we define ligand power (pow) as pow = $\log(\varepsilon)\log(1/K_A)$, where ε is the intrinsic efficacy of the ligand for the pathway and K_A is the equilibrium dissociation constant of the ligand-receptor complex. Therefore, ligand power reveals the relationship between ligands and pathways. Assuming $K_A < 1$, the powers of agonists ($\varepsilon > 1$), neutral antagonists ($\varepsilon = 1$), and inverse agonists ($\varepsilon < 1$) are positive, zero, and negative, respectively. The absolute value of ligand power indicates the strength of the ligand's influence on a pathway, and the sign indicates the direction of change. In other words, a ligand of a positive power activates the pathway, while a ligand of a negative power inhibits it. The absolute value of ligand power displays the extent to which the ligand activates or inhibits the pathway. When the ligand power is zero, the ligand is a neutral antagonist and does not affect the pathway directly. According to its quantification, ligand power is positively correlated with

affinity ($1/K_A$) for agonists but negatively correlated with affinity for inverse agonists. Given the sign of ligand power, these correlations are expected because a higher affinity leads to a greater absolute value of ligand power except for neutral antagonists. For neutral antagonists, the affinity does not influence ligand power, which is always zero. To sum up, ligand power can satisfactorily describe the pharmacological behavior of all kinds of ligands.

Since ligand bias is used to evaluate the differential impact of a ligand on several pathways, a ligand is unbiased if the ligand has the same power on all of the pathways affected by the receptor. Thereby, ligand bias can be quantified as Δpow or $\Delta(\log(\varepsilon)\log(1/K_A))$. A recent article [22] demonstrated that it is feasible to quantify ligand bias by using $\Delta\log(\varepsilon/K_A)$. This approach represents a significant advance in the quantification of biased agonism because it considers the constitutive receptor activity and eliminates the need to select a reference ligand.

However, the scale of $\Delta\log(\varepsilon/K_A)$ can produce counter-intuitive results in some circumstances. As stated in [22], $\log(\varepsilon/K_A)$ cannot differentiate between the two facets of a ligand which is an inverse agonist for pathway 1 ($\varepsilon_1 = 0.01$ and $K_{A1} = 10^{-6}$) and a neutral antagonist for pathway 2 ($\varepsilon_2 = 1$ and $K_{A2} = 10^{-4}$). Based on this scale, the bias of this ligand would be zero with respect to pathway 1 and pathway 2, yet it can be argued that this is not the case since the ligand clearly has differential effects. $\Delta(\log(\varepsilon)\log(1/K_A))$ can be viewed as an extension of this scale since it is also composed of the parameters ε and K_A in the Slack & Hall operational model [23]. Of note, $\Delta(\log(\varepsilon)\log(1/K_A))$ is the first scale consisting of a combination of intrinsic efficacy and affinity which can quantify the bias of all types of ligands, including agonists, neutral antagonists, and inverse agonists. This is owing to the ability of the Slack & Hall operational model [23] to consider constitutive receptor activity. By the definition of ligand bias here, a ligand is biased towards pathway 1 if its power on pathway 1 is above that on pathway 2. There are two

possibilities for this situation. Firstly, if its power on pathway 1 is positive, the ligand prefers pathway 1 to pathway 2. Secondly, if its power on pathway 1 is zero or negative, the ligand does not dislike pathway 1 as much as pathway 2. For the hypothetical ligand mentioned earlier in the paragraph for which $\Delta\log(\varepsilon/K_A)$ could not adequately quantify the bias, Δpow provides an intuitive analysis of ligand bias. Its powers on pathway 1 and pathway 2 are -12 and 0, respectively, which shows that it is an inverse agonist for pathway 1 and a neutral antagonist for pathway 2. Accordingly, Δpow correctly tells us that this hypothetical ligand is biased towards pathway 2.

Combining system bias and ligand bias

It is important to note that ligand bias should be assessed within the context of a particular signaling system: a receptor, various transducers, and various effectors (see above). For a different signaling system, the same ligand may have a different ligand bias. Therefore, we should compare the ligand-receptor complex with the free receptor in light of their functions in distinct pathways in the same signaling system so as to judge whether the ligand is biased or not with regard to these pathways.

By combining system bias with ligand bias, we can derive **signaling bias** (see Glossary) for different pathways. Signaling bias can be deemed as the preference of the combination of the signaling system and the ligand over some of the pathways. As a consequence, the signaling bias should be dependent on the system and the ligand. One way to evaluate signaling bias is to

compare the fractional functional responses for different pathways. It follows that signaling bias can be quantified as $\Delta\log(f)$, where $f=E/E_m$ denotes the fractional functional response for the pathway. Given the dependence of functional responses on ligand concentration, a more feasible approach is to quantify signaling bias separately for three situations, namely when the ligand is at very low, intermediate, or very high concentrations. When the ligand is at very low concentrations, f is approximately f_{basal} and signaling bias very similar to system bias. When there is a very high concentration of ligands, the equilibrium dissociation constant of ligand-receptor complex almost does not affect the functional responses for the pathways, and signaling bias is roughly $\Delta\log(\varepsilon\chi/(1 + \varepsilon\chi))$ based on the Slack & Hall operational model (with unit slope) [23]. For the ligand at an intermediate concentration, signaling bias is also influenced by ligand concentration and equilibrium dissociation constant in addition to ε and χ.

Provided that a particular signaling system is unbiased regarding two pathways, the total signaling bias will be determined by ligand bias and ligand concentration. In another scenario, it is also possible that ligand bias counteracts system bias and thus the resultant signaling bias is zero for some concentrations of ligands. Figure 3 illustrates how system bias and ligand bias contribute to signaling bias in some circumstances. The system bias is zero in Figure 3a, Figure 3b, and Figure 3c, so the signaling bias is dependent on ligand bias and ligand concentration. These graphs display how the concentration-effect curves are influenced by ligand power. In Figure 3d, Figure 3e, and Figure 3f, the system bias is not zero between the pathways with different basal fractional responses, but the signaling bias is zero when the different curves cross or merge. For example, in Figure 3d, the basal fractional response of Pathway 2 is higher than that of Pathway 5, showing that the system is biased towards Pathway 2 relative to Pathway 5. Nevertheless, the ligand is a neutral antagonist for Pathway 2 (ligand power = 0) and an agonist for Pathway 5 (ligand power = 8). According to the values of ligand power, this ligand

is biased towards Pathway 5 relative to Pathway 2. In this case, as the ligand concentration increases, the ligand bias eventually counteracts the system bias, so the two concentration-effect curves finally converge, showing that the signaling bias is zero at a sufficiently high concentration of the ligand.

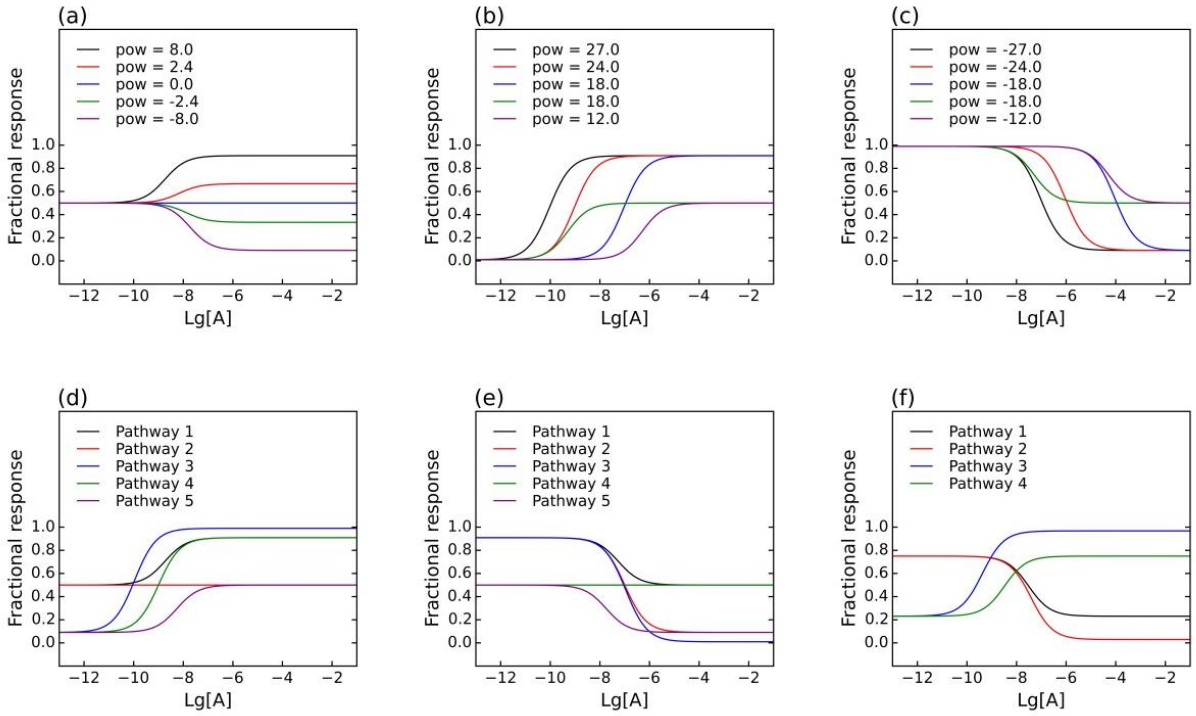

Figure 3. A selection of concentration-effect curves for the interaction between the ligand and the signaling system. (a), (b), and (c) show how the concentration-effect curves vary with ligand power. (a) all of five lines: $\chi = 1$, $K_A = 10^{-8}$. For ε, black line: 10, red line: 2, blue line: 1, green line: 0.5, purple line: 0.1. (b) all of five lines: $\chi = 0.01$. For K_A and ε, black line: 10^{-9} and 1000, red line: 10^{-8} and 1000, blue line: 10^{-6} and 1000, green line: 10^{-9} and 100, purple line: 10^{-6} and 100. (c) all of five lines: $\chi = 100$. For K_A and ε, black line: 10^{-9} and 0.001, red line: 10^{-8} and 0.001, blue line: 10^{-6} and 0.001, green line: 10^{-9} and 0.01, purple line: 10^{-6} and 0.01. (d), (e), and (f) display how ligand bias counteracts system bias at some ligand concentrations. $K_A = 10^{-8}$ for all lines in (d), (e), and (f). (d) For χ and ε, black line: 1 and 10, red line: 1 and

1, blue line: 0.1 and 1000, green line: 0.1 and 100, purple line: 0.1 and 10. (e) For χ and ε, black line: 10 and 0.1, red line: 10 and 0.01, blue line: 10 and 0.001, green line: 1 and 1, purple line: 1 and 0.1. (f) For χ and ε, black line: 3 and 0.1, red line: 3 and 0.01, blue line: 0.3 and 100, green line: 0.3 and 10.

Concluding remarks

Given the importance of constitutive receptor activity in the elucidation of ligand bias, we should try to measure the basal functional response of the signaling system for the pathways. When the basal response is too small to observe directly, it can be deduced by fitting the concentration-effect curves to the operational model [23]. Basal response tells us the original state of the signaling system before ligands are added. Only by comparing basal response with the functional response of the system with ligands can we really understand the functions of the ligands.

Signaling bias is a property of the signaling system in combination with the ligand, and it is influenced by system bias, ligand bias, and ligand concentration. System bias is an attribute of the signaling system, and it is determined by the components of the system, including the receptor, transducers, and effector molecules. Ligand bias is a property of the ligand within the context of the signaling system. With E_m being the reference, the system bias can be obtained by comparing f_{basal} of different pathways. With free receptor being the reference, we can calculate the powers of the ligand for different pathways. Ligand bias is the difference between these powers. If a ligand has the same power for these pathways, this ligand is unbiased with respect to these pathways. The proposed framework of signaling bias eliminates the need to

select another ligand as the reference, thus being applicable in a more general scope and in particular to inverse agonists.

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Competing interests

The authors declare no competing interests.

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Box 1

1. **The Black and Leff operational model of agonism** [20]

$$A+R \underset{}{\overset{K_A}{\rightleftharpoons}} AR \xrightarrow{K_E} E$$

The binding of the ligand to the receptor:

$$[AR] = \frac{[R]_T[A]}{K_A + [A]} \qquad (1)$$

With $K_A = \frac{[A][R]}{[AR]}$ and $[R]_T = [R] + [AR]$

A logistic equation is proposed for the transduction of receptor occupancy into effect:

$$E = \frac{E_m[AR]^n}{K_E{}^n + [AR]^n} \qquad (2)$$

The concentration-effect equation (3) results from the substitution of Equation 1 into 2.

$$E = \frac{E_m \tau^n [A]^n}{(K_A + [A])^n + \tau^n [A]^n} \qquad (3)$$

With $\tau = \frac{[R]_T}{K_E}$.

Constitutive receptor activity is not included in the model: The basal response is 0.

$$E_{[A]=0} = 0 \qquad (4)$$

The response at saturating concentration of the ligand:

$$E_{[A]\to\infty} = \frac{E_m \tau^n}{1+\tau^n} \qquad (5)$$

The potency of the ligand:

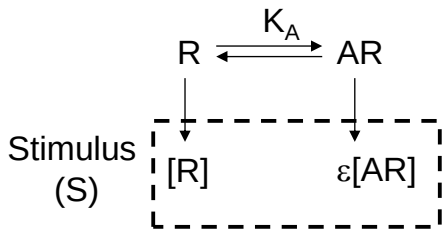
$$EC_{50} = \frac{K_A}{\left(2+\tau^n\right)^{1/n} - 1} \qquad (6)$$

The transduction coefficient for biased agonism

$\log\frac{\tau}{K_A}$, a single parameter combining efficacy and affinity values, was proposed as the transduction coefficient for biased agonism [17]. To cancel out system bias in a particular

signaling pathway, a reference compound is needed and $\Delta\log\frac{\tau}{K_A}$ is introduced. Finally, $\Delta\Delta\log\frac{\tau}{K_A}$ measures the bias of a ligand between two path)ways [17][19].

2. **The Slack and Hall operational model of agonism** [23]



The binding of the ligand to the receptor:

$$[AR] = \frac{[R]_T[A]}{K_A + [A]} \qquad (7)$$

With $K_A = \frac{[A][R]}{[AR]}$ and $[R]_T = [R] + [AR]$.

Both, the free receptor and the ligand-receptor complex produce a stimulus:

$$S = [R] + \varepsilon[AR] \qquad (8)$$

A logistic equation is proposed for the transduction of stimulus into effect:

$$E = \frac{E_m S^n}{K_E{}^n + S^n} \qquad (9)$$

The concentration-effect equation (10) results from the combination of Equations 7 to 9.

$$E = \frac{E_m \chi^n (K_A + \varepsilon[A])^n}{(K_A + [A])^n + \chi^n (K_A + \varepsilon[A])^n} \qquad (10)$$

With $\chi = \frac{[R_T]}{K_E}$.

Constitutive receptor activity is included in the model: The basal response is not 0.

$$E_{[A]=0} = \frac{E_m \chi^n}{1 + \chi^n} \qquad (11)$$

The response at saturating concentration of the ligand:

$$E_{[A]\to\infty} = \frac{E_m \varepsilon^n \chi^n}{1 + \varepsilon^n \chi^n} \qquad (12)$$

The potency of the ligand:

$$EC_{50} = \frac{K_A\left(\left(2\varepsilon^n\chi^n + \varepsilon^n + 1\right)^{1/n} - \left(\chi^n + \varepsilon^n\chi^n + 2\right)^{1/n}\right)}{\varepsilon\left(\chi^n + \varepsilon^n\chi^n + 2\right)^{1/n} - \left(2\varepsilon^n\chi^n + \varepsilon^n + 1\right)^{1/n}} \quad (13)$$

The scale for biased agonism

$\log\frac{\varepsilon}{K_A}$, a single parameter combining intrinsic efficacy and affinity values, was proposed as the scale for biased agonism [22]. No reference compound is needed to cancel out system bias in a particular signaling pathway. Finally, $\Delta\log\frac{\varepsilon}{K_A}$ measures the bias of a ligand between two pathways [22].

Glossary

Concentration-effect curve: the curve in an x-y plot of concentration-effect data where the x axis generally represents the logarithm of ligand concentration and the y axis shows the functional response of the signaling pathway.

Ligand bias: the difference in the ability of a ligand to activate the pathways in the signaling system.

Signaling bias: the difference in the activity level between different pathways in the signaling system without or with the ligand.

Signaling system: the receptor along with the transducer proteins it can interact with and the effector proteins downstream of those transducers.

System bias: the difference in the activity level between different pathways in the signaling system in the absence of the ligand.

5. Discussion

Receptors play vital roles in normal physiological processes in humans. They respond to a wide range of ligands and subsequently elicit downstream signal transduction. GPCRs make up a large superfamily of receptors in the membrane. For the GPCRs which reside in the cell membrane, their main function is to work as the bridge between the extracellular environment and the intracellular signaling machines. Because these GPCRs are located on the cell surface, it is more straightforward to design the drugs which can interact with them. Due to the important functions and localization of GPCRs, they are very attractive drug targets for the treatment of various diseases.

Mathematical models are very appropriate for the analysis of the correlation between drugs and their functional effects. The models can give some descriptors of how the drug interacts with the receptor in the context of the whole signaling system, such as the equilibrium dissociation constant of the ligand-receptor complex and the intrinsic efficacy of the ligand. Provided that there are two or more sites for ligand binding on the receptor, mathematical models can also tell us how the cooperativity between ligands quantitatively influences the function of the receptor.

Mathematical models are used for the description and explanation of experimental observations. Therefore, with the discovery of new phenomena with respect to GPCR function, the mathematical models of GPCR signaling need updating so as to reflect the up-to-date knowledge of drug-GPCR interactions. To the end, two new mathematical models of GPCR

function are proposed in the present thesis in order to account for the new finding concerning GPCR signaling, that is, GPCR oligomerization.

The oligomerization has been found for numerous GPCRs. It provides the GPCRs with other ways to perform their functions by affecting many aspects of GPCR signaling. For instance, there may be cooperativity between ligands which bind to different sites of one oligomer. Especially when the oligomer is composed of two distinct receptors, the oligomerization facilitates the crosstalk between the downstream signaling pathways which are respectively mediated by the two receptors. Under this circumstance, the ligand for one receptor can change the state of the signaling pathways downstream of the other receptor. Given that GPCR oligomerization has such a huge impact on the effects of GPCR-targeting drugs, the new mathematical models which are presented in the present thesis have great potential for the practical applications in the programs of drug development.

The heterodimer model in the present thesis is the first mathematical model which can quantitatively depict how the ligands alter the functional responses of the downstream signaling pathways mediated by a GPCR heterodimer. This model predicts the associations between the functional responses of two classes of pathways and the concentrations of two ligands which interact with the two different protomers of the GPCR heterodimer, respectively. This heterodimer model is derived from an existing operational model for the allosteric communications between two sites for ligand binding in a monomeric receptor.

An interesting experimental observation is that there may be a dominant receptor in the GPCR heterodimer. Specifically, of the two receptors in the heterodimer, one may produce stronger functional responses than the other. Our heterodimer model can delineate these phenomena

by assigning corresponding values to the parameters in the model. For instance, it is possible that within a heterodimer, one receptor is dominant over the other when no ligand is bound to the heterodimer. However, some ligands may reverse this functional dominance. By assigning the suitable values to the parameters of intrinsic efficacies, affinities, and so on in the heterodimer model, we can reproduce the same change in the functional dominance within the heterodimer by means of theoretical simulations. Given the huge complexity of the signal transduction mediated by heterodimers, this mathematical model can promote the comprehension of the functions of GPCR heterodimers.

A new mathematical model for GPCR homodimers is also proposed in the present thesis. This is an operational model for GPCR signaling with constitutive receptor activity. Because of the presence of constitutive receptor activity, this model can depict the actions of inverse agonists, neutral antagonists, and agonists. Moreover, the homodimer which this model is intended for has two binding sites for the ligands. Taking all of these factors into consideration, it can be expected that this model can produce a great variety of dose-response curves. Based on some possible values for the parameters in the model, theoretical simulations indeed generate multiple functional response curves with distinct shapes. This agrees with the complexity of experimental observations and shows that this homodimer model has the potential to be applied to the dissection of various experimental results.

This operational homodimer model can also assist in the understanding of some fundamental pharmacological concepts, such as the intrinsic efficacy of the ligand and the cooperativity between ligands. By comparing this operational homodimer model with the two-state dimer receptor model, we are able to identify the agreement between these two mathematical models in the explanation of the concepts of the intrinsic efficacy and the cooperativity.

Elucidating these pharmacological concepts from two different perspectives can help us to think about the real meanings of the intrinsic efficacy and the cooperativity. For example, these two models reveal the importance of the comparison between the free homodimer and the ligand-occupied homodimer in the determination of the intrinsic efficacy of the ligand.

This operational homodimer model can also delineate the concentration-dependent ligand preference with respect to several signaling pathways. As experimental observations suggested, the signaling pathways downstream of the β_2 adrenergic receptor varied from G-protein-dependent to G-protein-independent as the concentration of the ligand went up. In this operational homodimer model, the homodimer may be occupied by zero, one, or two ligands. The reasonable setting of the values of the parameters can enable the homodimer with different numbers of bound ligands to have discrepant preference regarding the G-protein-dependent and G-protein-independent pathways. In this manner, the operational homodimer model can give the dose-response curves which agree with the experimental results.

A new framework of biased signaling is built in the present thesis. This framework elucidates the relationships between system bias, ligand bias, and signaling bias. According to this framework, any signaling system has the basal response, although sometimes it is difficult to measure the basal response directly in the assays. The presence of constitutive receptor activity is an essential part of this framework since the basal condition tells us the state of the signaling system before the addition of the ligand. After the ligand is added to the signaling system, the ligand interacts with the system and alters the signaling output of the system. Therefore, the final functional response of the signaling system with the ligand is determined by the properties of the signaling system and the ligand. This provides the foundation for the construction of the framework of biased signaling.

The dissection of biased signaling can be illustrated using dose-response curves. The signaling bias can be calculated by means of the comparison between the fractional functional responses of different signaling pathways. When the concentration of the ligand is very low, the value of the dose-response curve is approximately the basal response of the signaling system. Under this condition, the signaling bias is almost equal to the system bias. As the concentration of the ligand increases, the signaling bias begins to be influenced by the ligand bias and the ligand concentration in addition to the system bias. After the ligand concentration becomes sufficiently high so that nearly all of the receptors are occupied by the ligand, the signaling bias is almost determined by the system bias and the ligand bias.

This framework of biased signaling not only provides a new insight into the contributing factors of biased signaling, but also gives the quantitative scales for system bias, ligand bias, and signaling bias. The concepts of system bias and signaling bias are straightforward to understand since they can be easily visualized by means of dose-response curves. However, the concept of ligand bias may be not so easy to comprehend. The quantitative scale for ligand bias is based on the intrinsic efficacy of the ligand and the equilibrium dissociation constant of the ligand-receptor complex. Therefore, it is possible that different combinations of the intrinsic efficacy and the equilibrium dissociation constant produce the same value of ligand power. In these cases, the ligand is unbiased according to the scale for ligand bias, although there are discrepant dose-response curves for different signaling pathways.

The effects of the ligand are determined by many factors, such as the intrinsic efficacy of the ligand and the affinity of the ligand for the receptor. Accordingly, the scale for ligand bias with respect to different signaling pathways must take these parameters into account. As a consequence, it is reasonable that a ligand with a ligand bias of zero may generate distinct

dose-response curves for different signaling pathways since the dose-response curves are influenced by these parameters.

The present thesis is made up of the three pieces of work mentioned above. These studies aim at providing new mathematical models or quantitative scales for the understanding of GPCR signaling. The heterodimer model and the homodimer model assist in the analysis of the functions of GPCR oligomers. Moreover, the quantitative scales for system bias, ligand bias, and signaling bias help us to dissect the biased signaling which is mediated by the GPCR. With the development of more advanced experimental techniques, the values of the parameters in these mathematical models will be increasingly accessible. It is expected that the models and scales in the present thesis will have a wide range of applications in pharmacology.

6. Conclusions

A mathematical model for GPCR heterodimers is proposed in the present thesis. This model quantifies the allosteric interactions between the two different protomers and the cooperativites between the ligands which are respectively bound to the two protomers within a GPCR heterodimer. The heterodimer model is derived from the operational model of allostery including constitutive receptor activity. The heterodimer model provides a good platform for the analysis of the functional crosstalk between distinct receptors which make up a heterodimer.

A mathematical model for GPCR homodimers is presented in the present thesis. A great variety of dose-response curves can be generated by assigning various values to the parameters in the model. These dose-response curves are consistent with a wide range of experimental observations. The definitions of some pharmacological concepts, such as the cooperativities between ligands and the intrinsic efficacy of the ligand, agree with those in the two-state dimer receptor model. The comparison between the two models assists in the understanding of these important pharmacological concepts. The operational homodimer model also explains the concentration-dependent ligand bias with respect to different signaling pathways.

A new conceptual framework for biased signaling is put forward in the present thesis. This framework clearly elucidates the definitions of system bias, ligand bias, and signaling bias for a signaling system and a ligand. In the absence of the ligand, the signaling bias is determined by the system bias. By contrast, the signaling bias is influenced by both the system bias and the

ligand bias after the ligand is added into the signaling system. Based on the framework, new quantitative scales for system bias, ligand bias, and signaling bias are proposed. The framework and the scales can help us to search for the unbiased or biased ligands with better therapeutic properties.

All of the three studies in the present thesis utilize operational models as the foundation for the analysis of GPCR signaling. Since there have been some well-established protocols for the investigations based on operational models, the new mathematical models for the receptor heterodimer and the receptor homodimer and the novel framework for biased signaling will have a wide range of applications in the pharmacological research.

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