Where is the bias?

Theoretical and experimental insights into the onset of ligand biased signaling by G protein coupled receptors.

Susanne Roth
Members of the Doctoral Examination Committee:

prof. dr. Martine Smit
VU University, Amsterdam, the Netherlands

prof. dr. Daniela Riccardi
Cardiff University, Cardiff, UK

dr. Henry Vischer
VU University, Amsterdam, the Netherlands

dr. Donald Ward
The University of Manchester, Manchester, UK

prof. dr. Hans Westerhoff
VU University, Amsterdam, the Netherlands

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door

Susanne Roth

geboren te Heppenheim, Duitsland
promotoren: prof.dr. F.J. Bruggeman
prof.dr. T.W.J. Gadella

copromotor: dr.ir. J. Goedhart
Summary

G protein-coupled receptors (GPCRs) are a versatile family of eukaryotic membrane spanning proteins that initiate cellular signal transduction. Usually, external ligands of various types are binding to a GPCR and activating it. Thereby, the receptor can integrate external stimuli in a ligand-dependent manner such that multiple signal transduction pathways are activated with different efficacies. This phenomenon called ligand biased signaling is known for many receptors of the GPCR family. It remains to be clarified to what extend the receptor contributes to signaling bias, and which role the signaling network plays in its propagation and modification. As GPCRs are targets of many established drugs, the knowledge of biased signaling is promising to lead to the development of improved drugs.

In this study, I investigated the onset and propagation of biased signaling with theoretical and experimental approaches. A mathematical model was established based on the recent understanding that ligand binding stabilizes distinct receptor conformations and thereby introduces bias in signaling. The model suggests that ligand bias can occur due to conformation-dependent ligand binding efficacies and that modifiers acting on allosteric binding sites additionally influence the ratio of these receptor conformations. Further experimental investigations of the onset of ligand bias were conducted with a cell line stably expressing the human external calcium sensing receptor (CaSR), that is known for its multifarious ligand binding and signaling. Using a FRET sensor of the intracellular G protein G_q, we observed its direct activation upon CaSR stimulation with various ligands, a read-out at the very beginning of the signaling cascade. In our real-time measurements we found several ligands initiating G protein activity that closely resembles downstream signaling read-outs that were described in literature about CaSR biased signaling. However, some ligands did not show bias in our assays in contradiction to published results of downstream read-outs. This indicates a modifying role of the signaling network downstream of the receptor. We conducted simultaneous measurements of G protein activation and intracellular calcium release in order to assess the dynamic co-activation of these two signaling events. In these results, the G_q activity and intracellular calcium mobilization show similar trends, but the precise co-variation of the signaling events depends on the ligand combinations used. These observations indicate that biased signaling results both from the receptor level and the downstream signaling network.

In summary, in this work advanced experimental tools and theoretical considerations were combined to investigate the onset and propagation of ligand biased signaling. This is a first step towards a deeper, network-level understanding of signal bias which might enable the targeted design and application of biasing drugs in future.
Nederlandse samenvatting

G-eiwit gekoppelde receptoren (GPCR’s) zijn een veelzijdige familie van eukaryote membraanoverspannende eiwitten die cellulaire signal transductie initiëren. Gewoonlijk binden liganden van verschillende type zich aan de GPCR en activeren het. Bovendien kan de receptor, afhankelijk van de ligand, verscheidene externe stimuli integreren, zodat meerdere signal transductie routes worden geactiveerd met verschillende doeltreffendheden. Dit fenomeen, dat vertekende ligand signalering genoemd wordt, is bekend voor vele receptoren uit de GPCR familie. Desalniettemin blijft het onduidelijk in hoeverre de receptor bijdraagt aan de signalerings vertekening en welke rol het signalerings netwerk speelt in de vermeerdering en verandering van de vertekening.

Omdat GPRC’s doelwitten zijn van vele gevestigde geneesmiddelen, is de kennis van vertekende signalering veelbelovend voor de ontwikkeling van verbeterde medicijnen.

Ik heb in dit onderzoek gekeken naar de start en voortplanting van vertekende signalering met zowel experimentele als theoretische technieken. Er is een wiskundig model ontwikkeld, gebaseerd op de huidige kennis dat ligand binding verschillende receptor configuraties stabiliseert en daarmee een vertekening introduceert in signalering. Het model suggereert dat ligand vertekende signalering kan ontstaan door de configuratie-specifieke ligand doeltreffendheden. Bovendien wordt de ratio van deze configuraties beïnvloed door modificeerders, die binden op de allostereische bindings locaties.

Verder is er experimenteel onderzoek gedaan naar de start van de vertekende ligand signalering met een cellijn die de menselijke externe calcium sensor receptor (CaSR) stabiel tot expressie had. CaSR is bekend om zijn verscheidenheid betreffende ligand binding en signalering. Gebruik makend van de FRET sensor van het tussencellulaire G eiwit Gq, observeerden we dat het eiwit direct geactiveerd werd na CaSR stimulering met verschillende ligands. Dit is een read-out in het begin van de signaliserings cascade.

In onze real-time metingen vonden we verschillende liganden die G eiwit activiteit initieerden dat erg leek op stroomafwaardse read-outs die waren beschreven in de literatuur over vertekende CaSR signalering. Sommige ligands echter, in tegenstelling met gepubliceerde resultaten, vertoonden geen vertekening in onze experimenten. Dit geeft een indicatie dat het netwerk, stroomafwaards van de receptor, een modificeerende rol heeft.

We deden simultane meetingen van G-eiwit activatie en tussen-cellulaire calcium afgifte om de dynamische co-activatie van deze twee signaleringsgebeurtenissen te
onderzoeken. In onze resultaten vertonen de Gq activatie en de tussen cellulaire calcium mobilisatie vergelijkbare trends. De precieze co-variatie van de signaliserings gebeurtenissen echter, hangt af van de gebruikte combinatie van ligands. Deze observaties indicieren dat vertekende signalering wordt veroorzaakt op zowel het receptor niveau als in het stroomafwaardse signalerings netwerk.

Samenvattend werden voor dit werk geavanceerde experimenterende technieken en theoretische overwegingen gecombineerd om de start en voortplanting van vertekende ligand signalering te onderzoeken. Dit is een eerste stap naar een dieper begrip van vertekende ligand signalering op het niveau van het netwerk. Eventueel zal dit in de toekomst het doelgerichte ontwerp en toepassing van medicijnen, die gebruik maken van de vertekende ligand signalering, mogelijk maken.
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CHAPTER 1

General Introduction
1.1 G protein-coupled receptors are environmental signal integrators

"No man is an island" is a famous quote of the 17th century poet John Donne. It expresses the connections that every person encounters with his or her social environment. In a broader biological sense, we can adapt this quote to "no living organism (or cell) is an island": a multitude of mechanisms have evolved that enable all organisms – unicellulars and multicellulars alike – to communicate with their environment. Such response mechanisms evolved because an appropriate reaction of an organism to its surroundings is essential for its survival.

For us, humans, information about the environment is encoded in stimuli such as sounds, light, temperature, tastes or smells. On a cellular scale, information is mostly transmitted in chemical form - e.g. as hormones. Molecular processes facilitate this exchange within and between cells. A cell carries out ‘signal transduction’ which includes the perception and integration of signals to initiate a reaction to the cell’s current environment. Signal transduction describes how cells in multicellular organisms exchange information and coordinate tasks. Signal transduction is realized by interacting proteins, forming huge protein-protein interaction networks. Proteins communicate with each other via a limited set of (dynamic) mechanisms, involving complex formation and protein modification. The complexity of signal transduction, therefore, emerges from the almost unlimited number of protein-protein interaction combinations that are possible, given the fact that the signaling network of a human easily involves hundreds to thousands of proteins [1].

Transmembrane proteins, receptors or channels, connect the outside of a cell with its interior. Some of them are activated by external signals that induce changes in their 3D structure, a mechanism that triggers activation of intracellular proteins. Intracellular proteins then activate each other in sequences, involving branches, loops, and feedbacks: networks. These activities are meant when we speak about ‘signal transduction’, ‘signal integration’ or ‘signal processing’ of perceived signals. Finally, the signals cause activation of downstream ‘response’ molecules, such as transcription factors that regulate gene activity, ultimately controlling cellular functions, such as motility, differentiation, proliferation or death [2]. In figure 1.1, such a ‘core’ signaling pathway is depicted.

Characteristics of the GPCR family

For eukaryotes, one important class of membrane-spanning receptors is the family of G protein-coupled receptors (GPCRs). A GPCR is characterized by a core structure of seven trans-membrane α-helices (TMs) and interaction with heterotrimeric G proteins. GPCRs can be activated by a plethora of signals, ranging from single
photons, small molecules (like ions or amino acids) to larger peptides (like hormones or neurotransmitters). The human genome encodes about 800 GPCRs [3]. Due to their versatility, GPCRs play important roles in physiology [4]. Their diversity, ubiquitous expression and function in physiology make them attractive for the pharmaceutical industry; estimations of approved drugs that target GPCRs range from 25 - 30% [4, 5] and the potential for drug discovery in this class of receptors is tremendous [6].

Human GPCRs can be classified in five main families according to their structure and ligand binding characteristics [6]: Rhodopsin, Secretin, Adhesion, Glutamate and Frizzled/Taste2. Members of these receptor families differ in the amino acid sequences of their extracellular N- and cytosolic C-termini. The cytosolic sites contain docking sites for intracellular molecules and sites for modifications, such as phosphorylation. The N-termini are involved in extracellular ligand binding. Most rhodopsin-like GPCRs have a short N-terminus and agonists bind to extracellular loops of the transmembrane (TM) domain. The N-termini of the adhesion GPCRs are however very long and diverse, with several binding domains for extracellular matrix molecules. The extracellular domains of the secretin and glutamate-like receptors are long as well and crucial for ligand interaction and receptor activation. Glutamate-like receptors have a N-terminus that is folded into two domains, forming a ligand binding cavity, called the Venus Flytrap (VFT) domain, due to its resemblance to the carnivorous plant. The ligand binding pocket in the VFT domain is conserved within this family of GPCRs. The transmembrane domains contain the binding sites that allow interaction with allosteric ligands [7].

Despite these differences, the shared structural motif of GPCRs is their core of seven transmembrane helices. In addition, they show conserved sequence motifs across all families [3, 8] and crystallographic investigations have revealed common conformational changes upon activation [9]. Conserved cysteine residues are thought to form stabilizing disulfide bridges [3].
It is now well established that GPCRs from the glutamate family are obligate dimers, covalently linked by disulfide bridges in the VFT domain and by those close to the TM domain [10]. Evidence exists that also receptors from other families form functional dimers. Whether they do so in vivo is still highly debated [9, 11, 12]; especially since studies with truncated glutamate-like receptors indicate that a GPCR monomer is sufficient to activate G proteins [13, 14]. Recent studies, largely based on fluorescence and bioluminescence techniques, investigated GPCR dimerization [12, 15–18]. Crystallographic studies discovered potential dimer interfaces in the TM domains of chemokine and opioid receptors [19], supporting the evidence that GPCRs form functional dimers [20]. Indeed, single molecule imaging revealed a dynamic equilibrium between GPCR monomers and dimers [21, 22], displaying the average lifetime of a rhodopsin-like GPCR dimer as well as the dimer-formation kinetics. Receptor dimerization may impact the functions of a GPCRs, for example its coupling to different G proteins [23, 24], and its signaling specificity, efficiency and allosteric regulation [25–28]. To make things even more complex, heterodimers have been shown to exist, suggesting GPCR crosstalk [27, 29].

**Beyond ligand binding: receptor activation and G protein interaction**

Recent advances in crystallography and of Förster Resonance Energy Transfer (FRET) studies in live cells allow detailed insight into the receptor structure and kinetics of ligand binding, receptor-G protein interaction and activation. Ligand binding appears to induce conformational rearrangements of the transmembrane helices relative to each other; as found by intramolecular FRET and by comparison of crystal structures of ligand bound versus free receptors [30–34]. These studies also revealed that GPCRs adopt multiple conformations, mainly in response to different agonists [35–38]. It is thought that ligand binding influences the energy landscape of the receptor, as well as its conformational variability at the intracellular side [39, 40].

On the cytoplasmic surface of rhodopsin-like GPCRs, an ‘ionic lock’ (formed by the highly conserved E/DRY motif on transmembrane helix 3 (TM3) and a glutamate residue on TM6) is ruptured during GPCR activation [34, 41]. This causes TM6 to move closer to TM5, opening a cavity for G protein binding [33, 34]. These shifts from inactive to active receptor conformations are fast, as determined by intramolecular FRET studies, ranging from several tenths of milliseconds for the β1 adrenergic receptor to about one second for the parathyroid hormone receptor (PTHR) [30, 31, 42]. The time constants for receptor activation are therefore receptor-type specific and are even influenced by the ligand binding. For example, biphasic ligand binding has been observed for the PTHR with a first, very fast interaction and a slower second binding step, interpreted as binding to the N-terminus and to the receptor core, respectively [43].

Serine/Threonine residues in the cytosolic C-terminus of GPCRs can be phosphorylated by protein kinase A and C (PKA and PKC, respectively) or G protein-coupled
receptor kinases (GRKs). This modification recruits β-arrestins to the receptor [4]. Arrestin binding was believed to uncouple the receptor from G protein signaling, induce receptor internalization, and subsequent signal desensitization. It has been shown, however, that β-arrestins induce signal transduction themselves by acting as scaffolds for other, G protein independent signaling molecules [44]. These alternative signaling routes add up to the multifunctionality and interconnectivity of GPCR signaling.

The hallmark of GPCRs is their ability to activate heterotrimeric G proteins with the intracellular loops of their TM domains. G proteins have diverse functions but a conserved structure, consisting of α- and βγ-subunits. The C-terminus of Ga interacts with the binding pocket that is exposed by an active GPCR [33]. Other structural features have also been identified as important for receptor-G protein coupling and selectivity; for example, the intracellular loop 2 of the receptor [45–47]. Receptor-G protein interaction kinetics is very fast and occurs on a similar timescale as receptor activation [48]. Whether G proteins ‘pre couple’ to receptors has not been demonstrated unambiguously [32].

In an inactive heterotrimeric G protein, the subunits are in close proximity and the Ga subunit is bound to GDP. Interactions with an active receptor induce conformational changes in the C-terminus of Ga, resulting in a rotation of its α-helical domain and the subsequent opening of the guanine binding pocket [33]. This causes the release of the bound GDP and its replacement by GTP. Thus, the GPCR acts as a guanine exchange factor (GEF). Next, the GTP-bound, active α-subunit and the βγ subunits change conformations, or even dissociate. Activation timescales of several hundred milliseconds were determined for Gs and Gi activation [4]. Both subunits are now able to activate downstream signaling pathways. Finally, a Ga protein can return to its inactive state when its GTP is hydrolyzed by a GTPase. When reassembled with Gβγ subunits, it is ready for a new activation cycle.

The external calcium sensing receptor (CaSR)

In the early 1990s, a GPCR has been identified that functions as a key regulator of whole-body, calcium homeostasis [49]. Terned after its primary physiological target, extracellular calcium, (Ca^{2+})_o, the calcium sensing receptor (CaSR) is primarily expressed within the chief cells of the parathyroid glands. It gets activated at high concentrations of (Ca^{2+})_o and suppresses the secretion of the calcium-retaining, parathyroid hormone (PTH), thereby maintaining the calcium concentrations in the physiological range of 1.1 - 1.3 mM [50].

The CaSR belongs to the family of metabotropic glutamate receptors [49]; it is an obligate dimer, coupled by cysteine residues of each monomer [51] and has large extracellular domains that form VFT-like structures. At least three to five (Ca^{2+})_o binding sites are estimated by computational prediction studies, explaining the high
cooperativity with Hill coefficients between 2 to 4 observed in various responses to \((\text{Ca}^{2+})_o\) exposure [52].

Further studies revealed the expression of the CaSR in various tissues, some of those are related to calcium regulation, e.g. kidney and intestines, and some not, e.g. liver, blood vessels and breast [53, 54]. The CaSR is a multifaceted receptor, activated by a broad range of ligands besides \((\text{Ca}^{2+})_o\). External calcium-independent CaSR activity is induced by di- and trivalent ions (\(\text{Mg}^{2+}, \text{Sr}^{2+}, \text{Gd}^{3+}\)), polyvalent cations like poly-L-lysine, polyamines, and aminoclycoside antibiotics [54–56]. Allosteric ligands can modify the affinity of the receptor for \((\text{Ca}^{2+})_o\), examples are amino acids and synthetic modifiers (calcimimetics and calcilytics). The CaSR therefore integrates several metabolic signals [57–59].

Three families of heterotrimeric G proteins couple to the CaSR: \(G_{\alpha q}\), \(G_{\alpha i}\) and \(G_{\alpha 12/13}\) [55]. Accordingly, signal transduction through different pathways occurs. CaSR-induced \(G_{\alpha q}\) activity results in the activation of the membrane-bound phospholipase C (PLC), which in turn hydrolyses phosphoinositides into 1,4,5-tris-phosphate (IP\(_3\)) and diacyl glycerol, resulting in intracellular calcium \((\text{Ca}^{2+})_i\) mobilization, and activation of protein kinase C (PKC) [60, 61]. Therefore, \((\text{Ca}^{2+})_i\) mobilization and IP\(_3\) accumulation are common signaling read-outs in CaSR activity studies.

PKC induces the phosphorylation of the threonine residue 888 (T888) in the cytoplasmic tail of the CaSR, thereby uncoupling the receptor from \(G_{\alpha q}\) signaling [62]. It has been shown that T888 phosphorylation is essential for the physiological CaSR function and influencing the dynamics of intracellular calcium oscillations [63, 64].

Another well-studied CaSR read-out is the phosphorylation of the extracellular-signal-regulated kinase (ERK) 1/2. The underlying mitogen-activated protein kinase cascade is induced presumably by converging \(G_{\alpha i}\) and \(G_{\alpha q}\) signaling [50], whereas it is hypothesized that signaling through \(G_{\alpha 12/13}\) influences the \((\text{Ca}^{2+})_i\) oscillation-pattern in the presence of amino acids [65]. Cellular shape changes have been observed after CaSR stimulation, termed ‘membrane ruffling’ [66], probably caused by \(G_{\alpha 12/13}\)-mediated Rho GTPases and kinases that lead to rearrangements of the cytoskeleton and formation of actin stress fibers [61].

The most common diseases associated with the CaSR are the hereditary familial hypocalciuric hypercalcemia (FHH) and autosomal dominant hypocalcemia (ADH), caused by loss- and gain-of-function mutations in the receptor, respectively [67]. Nevertheless, reduced CaSR expression has been reported in parathyroid and colorectal cancer, resulting in the loss of the growth-suppressing effects of high \((\text{Ca}^{2+})_o\) concentrations [50]. In breast cancer, however, the reverse is observed: an increased expression and a switch in G protein coupling, from \(G_{\alpha i}\) to \(G_{\alpha s}\). This switch results in an elevated secretion of parathyroid hormone-related peptide (PTHrP), which is considered as a driving factor of metastasis [50]. Thus, the CaSR seems to play both ontogenic and tumor suppressor roles, depending on its expression level in the target tissue.
1.2 Ligand biased signaling of GPCRs

The early view that GPCRs exist solely in an "on" or "off" state has been succeeded by the current view that the receptors are in a dynamic equilibrium of different conformations [68, 69]. In this recent concept, ligands induce shifts in the conformation equilibria and stabilize particular receptor conformations [36, 70–73]. Hereby, biases in signal transduction correspond to the activation of downstream pathways to different degrees in a ligand-combination dependent manner (figure A). This 'ligand bias' has been documented for several GPCRs, including the CaSR [70, 74–78].

The dynamic conformation-equilibrium concepts suggests that ‘biasing drugs’ can improve efficacy and reduce side effects if they can specifically target the signaling pathways associated to disease [79, 80]. Thus, understanding how ligand-ligand interactions at the level of a GPCR bring about biased signaling has been recognized as a major challenge in the search for novel drugs [81–83]. Agonist screenings have revealed the biasing effects of clinically relevant compounds [84, 85], and pharmacologic active compounds with modulated biasing effects were developed [86, 87].

Ligand bias is viewed as an intrinsic property of the receptor-ligand complex and has been associated to the free energy landscape of a receptor, indicating the stabilization of particular receptor conformations [88, 89]. Whether signal transduction bias is solely attributable to a single GPCR is not clear however, perhaps some of the biases are introduced downstream of GPCRs in the signaling cascade.

Intracellularly binding peptides and nanobodies can, in addition to external signals, affect the coupling of GPCRs to signaling molecules (reviewed in [86]), indicating that the intracellular state may be involved in ‘priming’ the cell to specific signaling pathways and can modulate the biasing effects of extracellular ligands. Ligand bias is therefore a complicated concept that we do not yet fully understand. With this thesis, I hope to contribute to an improved understanding of the mechanisms associated with ligand bias.
**1.3 Studying ligand biased signaling**

In most studies, ligand bias is observed at the level of read-outs downstream of a GPCR, such as the generation of second messengers, phosphorylation of signaling proteins, changes in the cellular structure or receptor internalization [66, 90–92]. Thus, the biasing effect of ligands is deduced from shifts in dose-response curves of these signaling responses [80, 93, 94], for instance from bias plots [78] (figure 1.2B).

Downstream read-outs are measured with various biochemical techniques, ranging from enzyme and immunoassays to luminescence-based assays and fluorescent sensors [95]. These methods are well established and applicable to a broad range of cellular systems. However, the limitation is that these read-outs are located downstream in the signaling cascade. Therefore, they are influenced by intertwining signaling pathways and do not solely reflect the bias induced at the initial signaling steps, at the level of a single GPCR, but they rather report the integrated bias of the interacting signaling network, including the receptor.

Imaging methods allow for the study of real-time signaling responses in single, living cells [96]. This is advantageous for determining dynamic signaling events, for example for oscillations and other temporal processes, such as transient activations or subpopulation behaviors – that occur asynchronously across individual cells. These responses are generally averaged out in population-based assays, and are therefore no
longer observable when studying cell populations. Since “seeing is believing” *, these optical techniques allow the direct study of protein-protein interactions, processes occurring during receptor activation or downstream signal transduction. With current techniques it is therefore possible to study responses to ligand binding directly at the receptor, at the level of G protein activation or further downstream [31, 32, 48, 97, 98]. Time-resolved measurements additionally inform us about the kinetics of (de-)activation processes in signaling and lifetimes of active signaling components; important information needed to understand the underlying biological mechanisms of signal transduction [99, 100].

Most of the above mentioned studies with single, living cells exploit Förster Resonance Energy Transfer (FRET) based sensors. Described by Förster in the early 1950ies [101], FRET is nowadays an established, frequently-used tool in cell biology [102]. The underlying concept of FRET is a non-radiative energy transfer from a donor fluorophore to an acceptor fluorophore; this requires a close proximity (<10 Å) of two fluorophores and labeling of the protein(s) of interest [97, 103]. Since the excitation spectrum of the acceptor overlaps with the emission spectrum of the donor fluorophore, the acceptor emits light when it is excited by a donor via FRET [102, 103]. FRET is typically quantified by the ratio of acceptor and donor fluorescence [97]. With the development of fluorescent proteins with various excitation and emission spectra, a palette of FRET-pairs with improved photophysical effects is available today. Most commonly used variants rely on cyan and yellow fluorescent protein pairs (CFP and YFP) [104, 105].

Stimulus-induced association and dissociation of signaling proteins can be studied by FRET techniques, e.g. ligand-receptor interactions or G protein-receptor interactions by intermolecular FRET [42, 43, 48]. Conformational changes in GPCRs or G proteins upon activation can also be measured with (intramolecular) FRET [30, 31, 106, 107]. Therefore, we can assess biased signaling at various nodes throughout the signaling network in single, living cells.

1.4 How mathematical models help in understanding signal transduction

The study of cellular signal transduction has for a long time been limited to the identification and description of the associated molecules and processes; the emphasis has not been on how they together give rise to functionality of the signaling networks. The identification and descriptive approach was, and remains, very successful: it brought us the knowledge about the connectivity of signaling networks that we have today [1]. The resulting network diagrams are static, however, whereas

* The title of a symposium of the European Molecular Biology Laboratories (EMBL), Heidelberg, in October 2015.
signal transduction is a highly dynamic process, consisting of temporal protein interactions. The qualitative description of signaling molecules is therefore not sufficient to understand dynamic signaling mechanisms. Similarly, we do not understand how a radio is working by just naming its components [108]: likewise, biological systems are clearly more than the sum of their parts [1].

Systems biology is shifting the focus towards an understanding of the behavior of molecular systems over time, including the underlying mechanisms, and relies heavily on mathematical models [109]. Systems biology is ideally suited to study the dynamics of signaling networks in terms of interacting signaling proteins [110].

A mathematical model, based on established biological knowledge and tailored to the research question of interest, can make predictions about the system behavior, given specific conditions (in silico experiments), and aid in the design of new experiments [111]. Ideally, quantitative, experimental data is then used to test if the model represents the biology adequately. In a repetitive cycle, knowledge gained from the experiments are ideally used to improve the predictive power of the model (see figure 1.3)[111]. Thereby, the mechanisms, involving protein-protein interactions, that underlie system behavior, such as dynamic signal transduction by a specific pathway, can be understood, including the effects of mutated proteins and external perturbations, such as drugs, growth factors, cytokines, etc. More conceptual mathematical models, in the absence of experimental data, can also provide insights into whether a particular hypothesis is realistic or which system behavior can be expected.

Technological advances, for example in fluorescence microscopy, have made the generation of time-resolved, quantitative data in the GPCR field feasible. Combined with a systems approach, new insights into the complexity of GPCR signaling and crosstalk can be gained, as illustrated by several examples, e.g.[112–114], indicating the enormous potential for adjusted therapy and specified drugs on the basis of the systems-biological understanding of biological processes [115, 116].
Figure 1.3: Iterative cycle between experimental and theoretical approaches in systems biology. By an iterative cycle of quantitative data generation, mathematical modeling, in silico predictions, experimental validation and the design of new experiments, new insights into biological questions and contradictory hypotheses are obtained. Important prerequisite is a close collaboration between experimenters and theoreticians. Adapted from [111] by the department ‘Systems biology of signal transduction’ of Prof. Dr. U. Klingmüller, DKFZ, Heidelberg, Germany.

1.5 Aim of this thesis

I have introduced GPCRs as versatile membrane receptors that are capable of signal integration. Their multifarious ligand binding and different signaling conformations allow for a variable activation of the downstream signaling pathways that they connect to, known as ligand bias. Recent experimental techniques do allow for the study of ligand bias and are believed to have great potential in the design of ‘biasing drugs’. The limitation of those studies is that the measured signaling read-outs are generally (far) downstream of the GPCRs of interest. It therefore remains unclear, in most cases, whether the observed ligand bias of downstream signaling read-outs can be attributed to the GPCR or, in addition or exclusively, to downstream signaling processes. This is a complication when one thinks about designing biasing drugs for GPCRs; perhaps those drugs should target downstream processes instead?

In this thesis, I report my studies on the occurrence of ligand bias at the level of a GPCR, as well as downstream, with theoretical and experimental approaches. Part of my work is motivated by recent results from crystallographic and kinetic studies indicating that GPCRs can adopt several active, signaling conformations that are stabilized by specific ligand combinations. I will show that a conformation-equilibrium model of a GPCR can explain ligand bias at the level of the receptor (Chapter 2). I used the CaSR, which is known to show biased signaling when exposed to different agonists, to study ligand bias experimentally. Using advanced fluorescent microscopy techniques, I will show the biasing influence of several (allosteric) agonists on CaSR-induced $G_{aq}$ activation in single, living cells (Chapter 3). $G_{aq}$ directly cou-
ples to the CaSR and is therefore one of the closest read-outs of receptor activity that one can use. My data shows similarities as well as differences to the biasing effects of those CASR ligands described in literature that were observed in downstream read-outs. To differentiate between CASR-induced and network-induced bias, I measured the propagation of signaling bias from \( \mathrm{G_{\alpha q}} \) activation to intracellular calcium mobilization, using a second fluorescent reporter, R-GECO1 (Chapter 4). By simultaneously measuring these fluorescent read-outs, I found evidence that both the CASR and the downstream signaling network determine ligand bias. I also review recent systems biology approaches to signal transduction and how those can be applicable in the GPCR field (Chapter 5), since in the GPCR field systems biology is not yet used frequently. Finally, I discuss my findings in a broader context and propose that we should view a single GPCR as a signaling network by itself (Chapter 6). I hope that my thesis helps to further strengthen the concept of ligand bias by GPCR-activated signaling network by offering a systems biology perspective.
A conformation-equilibrium model captures ligand ligand interactions and ligand biased signaling by G protein-coupled receptors

In collaboration with:
Frank J. Bruggeman

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Abstract

G protein-coupled receptors (GPCRs) are a versatile, important class of cell-surface receptors. GPCRs occur in different conformations that exist in a dynamic ligand sensitive equilibrium. These conformations vary in their affinities for intracellular signaling proteins and initiate signaling via different intracellular routes. The binding of extracellular ligands and allosteric ligand-ligand interactions shift conformation equilibria to cause biased signaling. Here, we present a mathematical model that describes the effects of ligands on the conformation equilibria of GPCRs. Our extended Monod-Wyman-Changeux model describes the receptor as shifting between active and inactive receptor conformations under the influence of extracellular ligands. For each receptor conformation, the intracellular domain of the receptor can attain alternative domain conformations that differ in their affinity for intracellular signaling proteins. At the extracellular domain, the model can accommodate different mechanisms for allosteric ligand-ligand interactions that induce shifts in receptor and domain conformation equilibria. We use the model to study ligand biased signaling and how ligand affinity, ligand sensitivity, and maximal signaling output depend on allosteric ligand-ligand interactions.

Keywords: G protein-coupled receptors; ligand biased signaling; MWC model; cooperativity; allostery

2.1 Introduction

Structural [117–119] and kinetic [120–122] studies indicate that GPCRs exist in an equilibrium of different conformational states. Different intracellular signaling proteins are activated by dedicated receptor conformations [119, 123]. Changes in receptor-conformation abundances are triggered by the binding of extracellular ligands. Conformation changes involve rearrangement of transmembrane helices [124, 125] that sensitise the cytoplasmic domain of the receptor for specific intracellular signaling proteins. In this manner, bias is introduced to particular signaling routes [126–128] by the binding of different ligands, their combinations, and allosteric interactions [126, 129, 130].

The extracellular domains and ligand-binding pockets of GPCRs show a large variability in sequence and structure [131] indicating the great ligand variety across the family of GPCRs. In contrast, the transmembrane domain and intracellular domain of GPCRs is more conserved [132]. This suggests that the structural rearrangements in the transmembrane and cytoplasmic domain during receptor activation likely follow similar mechanisms across the family of GPCRs [133]. Therefore, a general model explaining ligand-induced conformational changes of GPCRs is a relevant problem to address.
Whether functional GPCRs occur in the plasma membrane as monomers, (hetero- or homo-) dimers or even oligomers has been under discussion for a long time [134]. Several studies support the view that functional GPCRs exist either as homo- or heterodimers [135, 136] and that two GPCR monomers together form a binding pocket for intracellular signaling proteins [137]. The binding of extracellular ligands and their allosteric interactions can then induce conformational changes in the receptor that trigger cooperative, (de-)stabilising monomer-monomer interactions or sensitisation of the dimer to particular intracellular signaling proteins [137, 138].

The study of the kinetic properties of cooperative, oligomeric proteins, which exist in different conformations and function at thermodynamic equilibrium, has a long history in biochemistry [139–142]. Such models are ideally suited to study signaling receptors, such as GPCRs, that display shifts in conformation equilibria upon ligand binding and that do not rely on free-energy input to display conformation changes [141]; in contrast to, for instance, receptor tyrosine kinases and many transcription factors that change to an active conformation upon phosphorylation. In the GPCR field, mathematical models have been used to fit experimental data to assess ligand affinity and allosteric ligand ligand interactions [143–145]. Those models are generally simplified representations of the conformation equilibria of GPCRs; the GPCRs are not considered as dimers with different (global) receptor conformations and (local) intracellular domain conformations. As a result, those models are of limited applicability when used in combination with experimental methods that address the relation between receptor structure, conformation dynamics, and function, such as is currently done using NMR and crystallography [118, 146]. Thus, conformation-equilibria models of GPCRs based on a more mechanistic description of receptor-ligand interactions and shifts in conformation equilibria are becoming more relevant.

Here we present a general model of the ligand-induced conformational changes of GPCRs; it is an extension of the Monod-Wyman-Changeux (MWC) model [139]. The model considers the GPCR as a dimer that exists in an equilibrium between global receptor conformations and local intracellular binding domain conformations. These equilibria are influenced by ligand binding and allosteric ligand ligand interactions. The model we present can accommodate a great variety of monomers that differ in the number of ligand binding sites and allosteric ligand ligand and ligand-modifier interactions. We study how this model accounts for ligand bias of active signaling conformations in terms of their ligand affinity, ligand sensitivity, and maximal signaling output.
2.2 Results

A conformation-equilibria based mechanism of GPCR activity

Figure 2.1 shows the basic GPCR mechanism that we study in this paper. The GPCRs are considered as homodimers. The model can be extended to deal with heterodimers, or even oligomers, but we do not consider this here. Each monomer has an extracellular ligand-binding domain and an intracellular binding domain for downstream signaling proteins. The extracellular binding domain can accommodate binding of multiple identical or different ligands, with or without allosteric interactions. The exact details depend on the GPCR of interest, but in principle the model can accommodate all possible allosteric interaction diagrams. The intracellular domain is only functional if the monomer interacts with another monomer in a dimeric complex. Accordingly, we limit this study to dimers. The intracellular binding domain of the dimer binds intracellular proteins, such as G-proteins or β-arrestins, provided it is in the correct conformation.

In the model, signaling by the receptor occurs because the binding of extracellular ligands induces changes in the equilibria that exist between different active receptor conformations. As a consequence, one or several conformation states increase in abundance upon the binding of one ligand (at the expense of others). Subsequently, the intracellular signaling proteins that have a high affinity for those states bind preferentially and signaling is biased to the downstream networks that they connect to. Thus, this mechanism naturally leads to ligand bias. Other ligands can subsequently introduce an additional shift in the conformation equilibria; for instance, via allosteric
The model that we consider distinguishes two kinds of conformation changes of the GPCR. Receptor conformation changes concern changes in the conformation of the entire dimer, whereas domain conformation changes apply only to intracellular binding domain changes. The latter can occur while the global conformation stays the same. Thus, at each (global) receptor conformation state, the (local) domain conformation can have various states that differ in their binding affinities for intracellular signaling proteins. This is the mechanism in this model that leads to ligand bias. Extracellular ligands can shift both the receptor and domain equilibria. This is where this model differs from existing models of cooperative proteins based on the MWC model, and of GPCRs in particular [143–145]. These models do not consider conformations of the intracellular binding domain in addition to global receptor conformations.

We distinguish between receptor and domain conformations to be able to capture a condition that is typically observed in experiments. In the absence of any ligands, there typically exists no, or very little, basal signaling activity. This situation we describe as the receptor occupying an inactive (global) receptor conformation state. Following the MWC terminology, this state corresponds to the "tensed" $T$ state of the receptor. As a result, all the (local) domain conformations of the $T$ state are not active. To allow for ligand bias, we require an active (global) receptor conformation – the "relaxed" $R$ state – for which the (local) domain conformations can each activate another intracellular signaling route. This is why we introduce the concept of different domain conformations for the intracellular binding domain of the receptor. The actions of ligands introduce shifts in the receptor conformations from $R$ to $T$ and vice versa, as well as in the conformations equilibria of the intracellular domain conformations.

From a thermodynamic perspective, we consider the GPCR at a state of thermodynamic equilibrium (i.e. in the absence of any sustained free-energy input). GPCRs operate in a state of thermodynamic equilibrium or close to it. In contrast to, for instance, receptor tyrosine kinases, where free energy input, e.g. through ATP hydrolysis, is used to drive conformation changes. We assume intra-receptor dynamics to be fast, relative to changes in extracellular ligand and intracellular target protein concentrations, such that on the time scales of appreciable ligand and target-protein concentration changes the receptor instantaneously relaxes to equilibrium concentrations of its conformations.

Different approaches exist to describe oligomeric proteins that exist in such quasi-equilibrium conformation states: the most famous approaches, which differ in their simplifying assumptions for the conformation change transitions, are the Adair-Klotz model [147, 148], the Koshland-Nemethy-Filmer (KNF) model [142], and the Monod-Wyman-Changeux [139] (MWC) model. In addition to the KNF and MWC model, which both simplify the conformation equilibria transitions, a model without those simplifying assumptions can also be considered [149]. We limit this study to the MWC model, but the mechanism outlined in Figure 1 can also be captured by the other modeling approaches. We focus on the MWC model because this
model contains a limited number of kinetic parameters and only concerns inactive versus active receptor conformations, and not on intermediate conformations in contrast to the KNF model. The latter is relevant because experimentally the signaling activity of alternative (active) receptor conformations is very hard to distinguish.

Figure 2.2: A particular model of a GPCR following the mechanism outlined in Figure 2.1. Two receptor conformations, $R$ and $T$, are distinguished. Two domain conformations can occur at the different receptor states (i.e. $R_1$, $R_2$ for $R$ vs $T_1$ and $T_2$ for $T$). We consider an extracellular binding domain with two binding sites for a ligand, $S$ (yellow circles). In the main text, we describe how the fractions of the different GPCR states can be expressed in terms of the ligand concentration, ligand affinity, and the conformation-equilibrium constants ($\ell_T$, $\ell_R$, and $L$).
Translation of the GPCR conformation-equilibria mechanism into a mathematical model

In Figure 2.2, the GPCR mechanism is shown that we consider, and will be extended in various directions, in the next sections. The GPCR has two global receptor conformations, $R$ and $T$, and two local domain conformations that can occur regardless of the global receptor conformations. The domain states for the $R$ states are denoted by $R_1$ and $R_2$ and for $T$ we have $T_1$ and $T_2$. We consider the $T$ states as inactive. We believe this to be a relevant scenario for most GPCRs. Specific GPCRs may then differ in the number of receptor and domain conformations, which can be considered as model extensions, but the essence remains the same: global active versus inactive receptor conformations and local domain conformations that are intracellular signaling protein specific.

A specific receptor state is characterized by three features: its global receptor conformation ($R$ or $T$), its domain state (if globally in $R$ state then $R_1$ or $R_2$ else $T_1$ or $T_2$), and the number of ligands $S$ bound, e.g. $R_2S_4$, and if two different ligands occur (e.g. $X$ and $Y$) then one could have $T_1X_2Y_4$ for instance. This logic expands without problems if more receptor conformations or ligands are considered. Note that the MWC model rules out mixed global conformation states, a dimer with one monomer in the $T$ state and the other in the $R$ state is excluded. We return to this point later.

In the appendix, we show how the concentration fractions of the receptor states can be expressed in terms of the concentrations of ligands and modulators, the equilibrium constants of the conformation equilibria, and the allosteric interactions between ligands and modulators. There we show that a relevant function to consider is the so-called partition function of the receptor system defined in equilibrium statistical physics [150]. A partition function is used to describe the receptor fractions that occupy different states. We deliberately discern the partition functions of the monomers from the partition function of the dimerized receptor as this simplifies the model greatly.

The partition functions of the monomers are dependent on the precise configuration of their binding sites and allosteric interactions. It can be used to express the monomer fractions occupying a particular ligand-bound conformation state in terms of experimentally-accessible kinetic parameters. It contains the dissociation constants of the different binding sites and the (allosteric) interaction coefficients that modulate the affinity of particular binding sites when others are occupied; for instance, we can have for the monomer partition function,

$$z_{i,j} = 1 + \frac{S}{K_{i,j}^1} + \frac{S}{K_{i,j}^2} + \frac{S^2}{\alpha_{i,j}K_{i,j}^1K_{i,j}^2}$$

(2.1)

with $i = \{r, t\}$, $j = \{1, 2\}$. Thus, $z_{r,1}$ denotes the partition function of the $R_1$ state. Equation 2.1 indicates that this monomer has two binding sites for the ligand $s$ occurring at concentration $S$ that have an allosteric interaction (see Supplementary
Information for a quick introduction). In case of \( z_{r,1} \) we obtain the following interpretation; the two binding sites have dissociation constants \( K_{1,r,1} \) and \( K_{2,r,1} \) and \( \alpha_{r,1} \) describes the allosteric interaction between the sites. Positive allostery occurs when \( \alpha_{r,1} < 1 \) as then the affinity of the monomer for \( s \) increases when one of the sites is already occupied. The fraction of the \( R_1S \) state (with site 2 occupied) equals \( R_1S / R_{1,T} = \frac{1}{z_{r,1} K_{2,r,1} S} \) (see Supplementary Information).

The advantage of our formulation is that the partition function of the entire receptor, which considers all monomer-monomer interactions and monomer binding states, can be expressed in terms of partition function of its two interacting monomers. As a result, we obtain a general description of GPCRs in terms of their monomers (explained below), regardless of the precise binding properties of these monomers. Hence, monomers act as modules in our approach, each described by its own partition function that characterizes their configuration of binding sites. This modularity requires the following assumptions: i. The monomers only influence each other’s conformation, and ii. global conformation changes occur in symmetry (the MWC assumption). As a result, the binding affinity of one monomer is only determined by the occupancies of its own binding states and not by the binding states of its partner monomer with which it interacts. Then, the monomers can be considered as modules. This does not mean that the monomers act independently; they do cooperate to give rise to concerted conformation changes but they do not engage in allosteric signal (de-)sensitization of their binding sites without the occurrence of a conformation change. The fact that monomers can be considered as modules makes this description highly attractive and easily extendible to different GPCRs that differ in the number of extracellular binding sites and allostERIC interactions.

To illustrate this modularity, we consider the \( R_1S_4 \) state of Figure 2.2 as an active signaling state of the receptor; say, it can activate a particular trimeric G-protein of interest. Using the theory outlined in the appendix, we can express the receptor fraction of the \( R_1S_4 \) state as,

\[
\frac{R_2S_4}{G_T} = \frac{\left( \frac{S^2}{\alpha K_{1,r,1} K_{2,r,1}} \right)^2}{Z} \tag{2.2}
\]

\[
Z = (z_{r,1})^2 + \ell_R (z_{r,2})^2 + L \left( (z_{t,1})^2 + \ell_T (z_{t,2})^2 \right) \tag{2.3}
\]

the \( z_{i,j} \)'s are the partition functions of the monomers in receptor conformation \( i = \{r, t\} \) and domain conformation \( j = \{1, 2\} \), and \( G_T \) denotes the total receptor (dimer) concentration. Equation 2.3, \( Z \), is the partition function of the entire receptor expressed in terms of equation 2.1, the partition function of any of the monomers. The \( L \) coefficient gives the ratio of the global receptor conformations \( T/R \), the \( \ell_R \) gives the ratio of the domain \( R \) conformations, i.e. \( R_2/R_1 \), and \( \ell_T \) does the same for the domain \( T \) conformations, \( T_2/T_1 \). The \( L, \ell_T, \) and \( \ell_R \) constants are equilibrium constants of the conformation equilibria; below we will show that they can be influenced by ligand binding and allostERIC interactions. The modularity is illustrated by the fact
Conformation-equilibrium model of GPCRs

that $Z$ can be expressed in terms of the $z_{ij}$’s, the monomer characterization in terms of its binding site affinities and the allosteric interactions. In the Figure 2.3, we outline different monomer binding and allosteric-interaction diagrams.

<table>
<thead>
<tr>
<th>Description</th>
<th>Partition function, $z_{ij}$, $i = {r,t}, j = {1,2}$</th>
<th>Pictogram</th>
</tr>
</thead>
<tbody>
<tr>
<td>two identical binding sites</td>
<td>$z_{ij} = \left(1 + \frac{S}{K_{S_{ij}}}\right)^2$</td>
<td><img src="image1" alt="Diagram" /></td>
</tr>
<tr>
<td>two identical binding sites with allosteric interaction</td>
<td>$z_{ij} = 1 + 2\frac{S}{K_{S_{ij}}} + \frac{S^2}{\alpha K_{S_{ij}}^2}$</td>
<td><img src="image2" alt="Diagram" /></td>
</tr>
<tr>
<td>two signal binding sites with a modifier binding site</td>
<td>$z_{ij} = \left(1 + \frac{S}{K_{S_{ij}}}\right)^2 \left(1 + \frac{M}{K_{M_{ij}}}\right)$</td>
<td><img src="image3" alt="Diagram" /></td>
</tr>
<tr>
<td>two signal binding sites and a modifier binding site with allosteric interactions</td>
<td>$z_{ij} = 1 + 2\frac{S}{K_{S_{ij}}} + \frac{S^2}{\alpha K_{S_{ij}}^2} + \frac{M}{K_{M_{ij}}} + \frac{S \cdot M}{\eta_{ij} K_{S_{ij}} K_{M}} + \frac{S^2 \cdot M}{\alpha \eta_{ij} K_{S_{ij}}^2 K_{M}}$</td>
<td><img src="image4" alt="Diagram" /></td>
</tr>
<tr>
<td>signal and competitive inhibitor binding on two binding sites</td>
<td>$z_{ij} = 1 + 2\frac{S}{K_{S_{ij}}} + \frac{S^2}{\alpha K_{S_{ij}}^2} + \frac{S \cdot X}{K_{S_{ij}} K_{X}} + \frac{S \cdot X}{K_{X}^2}$</td>
<td><img src="image5" alt="Diagram" /></td>
</tr>
</tbody>
</table>

Figure 2.3: Examples of partition functions of monomers that differ in the nature of binding sites. The monomer partition functions, $z_{ij}$, can be substituted in the partition function $Z$ for the entire GPCR (see main text). $S$ and $M$ denote a signal and a modifier concentration respectively. The $\alpha$ and $\eta$ coefficients are (allosteric) interaction coefficients between binding sites. The $K$ coefficients are all dissociation constants.

So far, we assumed that the conformation changes of the intracellular domain of receptor display so-called concerted symmetry, i.e. if one of the monomers in a dimer changes domain conformation then its partner does the same. Accordingly, mixed local conformations such as $R_1 R_2$ or $T_1 T_2$ do not occur, only $R_1 R_1$ and $R_2 R_2$. (If we would have written the partition function as $Z = (z_{r,1} + \ell_{R} z_{r,2})^2 + \ell_{T} (z_{t,1} + \ell_{T} z_{t,2})^2$ then we would allow for mixed local states.) The MWC model, by definition, already excludes mixed global conformation states, i.e. $TR$ and $RT$.

In the next sections, we show how ligand binding and ligand ligand interactions, as expressed in the partition functions of the monomers ($z_{ij}$’s), induces conformation changes of the receptor (changes in $T/R$, $R_2/R_1$, and $R_2 S_4/R_2 S_1$) and how this provides the mechanism for ligand biased signaling.
Incorporation of allosteric modifiers

Modifiers, as opposed to signals, are ligands that can bind to the extracellular binding domain of the GPCR but cannot induce signal transduction without the presence of another ligand, a “signal”. Signals can invoke signaling by themselves. In the appendix, we show that if modifiers do not show allosteric interactions with signals or other modifiers that then the partition functions of their binding sites can be subsumed in the equilibrium constants for the receptor and domain conformations, respectively \( L, \ell_R \) and \( \ell_T \); for instance,

\[
L' = L \left( \frac{1 + \beta}{1 + \gamma} \right)^2
\]  
\[
\ell'_R = \ell_R \left( \frac{1 + \beta_R}{1 + \gamma_R} \right)^2
\]  
\[
\ell'_T = \ell_T \left( \frac{1 + \beta_T}{1 + \gamma_T} \right)^2
\]

Modifiers that affect the conformational equilibria of the receptor can either act as activators by shifting the equilibrium to particular active states or as inhibitors by stabilising inactive states. In the equations for the modified equilibrium parameters \( L' \) and \( \ell'_R \), \( \beta \) represents an inhibitor (it pushes the receptor to the global T state) and \( \gamma \) is an activating modifier with respect to the \( T_1 \) and \( R_1 \) local conformations. An influence on \( L \) is an important mechanism to shift the receptor from the inactive \( T \) state, in the absence of a signal, to the active \( R \) states when the signals are present. Since, \( L = T/R \) a decrease in \( L \) via a modulator or signal would increase the receptor fraction in the \( R \) states. If, in addition, the same, or another, ligand affects the domain conformation equilibrium constant \( \ell_R \) then ligand bias can be introduced. An increase in \( \ell_R \) favors signaling via the \( R_2 \) state at the expense of signaling via the \( R_1 \) state. This again shows the importance of distinguishing between the receptor and domain conformations in this model of GPCR signaling.

Other than shifting conformational equilibria, modifiers can also affect the binding properties of the receptor directly through allosteric interactions with signals (allosteric modifier) or through competitive binding (competitive modifier). Allosteric modifiers can either have a stimulating or inhibiting influence on signaling; and even both if multiple receptor states are active in signaling but respond in an opposite manner to this modifier. A competitive modifier typically inhibits signaling if it competes with a signal. Such modifier activities can be incorporated into the partition functions of the monomers (Figure 2.3).

**Shifts in global and local conformation equilibria cause ligand bias**

The response of a GPCR to the binding of a signal or a modulator involves three sequential conformation changes. Firstly, the conformation equilibrium between the
active "R" conformation and the inactive "T" conformation shifts. Indeed in our model this phenomenon occurs and this is illustrated by the following equation,

$$\frac{\text{total } R \text{ state concentration}}{\text{total } T \text{ state concentration}} = \frac{\frac{1}{L} (z_{r,1})^2 + \ell_R (z_{r,2})^2}{\frac{1}{L} (z_{t,1})^2 + \ell_T (z_{t,2})^2}$$

(2.7)

If the domain conformations have different affinities for the ligands, such that their partition functions are not the same (i.e. $z_{r,1} \neq z_{r,2} \neq z_{t,1} \neq z_{t,2}$), then binding of the signal causes a shift in the ratio of active over inactive conformations. On top of that, the $L$ and $\ell$ coefficients, as well as the ligand dissociation constants, can be functions of modulators. Thus, also modulators can induce receptor conformation changes.

Secondly, the fractions of the different domain conformations of the active $R$ state can change in response to ligand binding. In terms of our model, we obtain,

$$\frac{\text{total } R_1 \text{ dimer state concentration}}{\text{total } R_2 \text{ dimer state concentration}} = \frac{\frac{1}{L} (z_{r,1})^2}{\frac{1}{L} (z_{r,2})^2}$$

(2.8)

Also this ratio varies with the signal concentration, provided the domain conformations have different affinities for the same signal, and with the modifier concentration. The modifier concentration can influence $\ell_R$ or influence signal affinity via a competitive binding or allosteric interaction with signal binding sites.

Thirdly, the ratio of the active state concentrations can change, indicating a mixed conformation and affinity effect,

$$\frac{R_1 S_4}{R_2 S_4} = \frac{1}{\ell_R} \left( \frac{\alpha_{r,2} K_{1,r,2} K_{2,r,2}}{\alpha_{r,1} K_{1,r,1} K_{2,r,1}} \right)^2$$

(2.9)

We note that this ratio equation is not a function of the signal concentration. As a consequence, a constant bias occurs when modulator concentrations are held fixed or if no modulators play a role. Thus, signal bias only occurs due to a modulator that acts in addition to a signal in our model. This is a consequence of our assumption that the binding affinities on one monomer are independent of the binding state of its partner monomer. This is an experimentally testable prediction of our model.

The hierarchy of the three conformation changes is illustrated with a numerical example in Figure 2.4.
Figure 2.4: Conformation changes of active states as function of signal and modifier concentrations. A) The dependency of $R_1 S_4$ on ligand concentration $S$. The inset depicts the fractions of the other $R_1$ states $R_1 S_0$ (blue), $R_1 S_1$ (purple), $R_1 S_2$ (yellow), $R_1 S_3$ (green). B) Fraction of $R$-state as function of the total receptor concentration. Blue, solid line: $L = 50$. The equilibrium between $R$ and $T$-states in the absence of ligand can be shifted towards the active conformation by modifying the $L$-coefficient, corresponding to a higher basal activity of the receptor (dashed, purple line, $L = 1$). C), D) Fraction of $R_1$ and active state $R_1 S_4$ as function of the total receptor concentration in the $R$-state, respectively. E) The ratio of $R_2 S_4$ to the active conformations can be influenced by a modifier acting on the equilibrium constant $\ell_R$. Thus, a switch in local conformations is obtained at high modifier concentrations, F) $R_2 S_4$ and $R_1 S_4$ are depicted in blue and purple, respectively, as function of the signal concentration. Parameters are listed in the appendix.

Ligand biased signaling illustrated with a bias plot

GPCRs display ligand biased signaling indicating that depending on the identity of the ligand, or the precise combination of ligands present, the GPCR activates different intracellular signaling routes. Ligand bias at the level of the receptor implies that particular receptor states linked to specific signaling routes are increased in fraction more than others when particular ligands are added. The influence of biasing modifiers is schematically depicted in figure 2.5A: a modifier can "push" an equilibrium to a different signaling state as was discussed above (equation 2.9).
Typically, bias plots are used to illustrate the signaling bias of one ligand in experiments [126, 151]. The level of one signaling route normalized by its maximal level is plotted as function of the same measure for an alternative signaling route. We illustrate such a plot in Figure 2.5B for the two active states, $R_1S_4$ and $R_2S_4$, of the GPCR in our model. In this figure we plot $\frac{R_2S_4}{\max(R_2S_4)}$ as function of $\frac{R_1S_4}{\max(R_1S_4)}$. The value of $\max(R_1S_4)$ equals the maximum level of the $R_1$ conformation, which equals $(z_{r,1})^2/Z$ (and the same for $\max(R_2S_4)$). In the Supplementary Information, we derive these normalised signalling responses. For instance, in the presence of a modifier
with concentration $m$ we obtain,

$$\frac{R_1S_4 + R_1S_4M}{\text{max}(R_1S_4 + R_1S_4M)} = \left( \frac{\eta_{r,1}^2 \eta_rK_{1,r,1}K_{2,r,1}K_{m,r,1}}{\eta_{r,1}^2 \eta_{r,1}^2 K_{1,r,1}K_{2,r,1}K_{m,r,1}} + \frac{S^2}{\eta_{r,1}^2 K_{1,r,1}K_{2,r,1}K_{m,r,1}} \right)^2$$

$$z_{r,1} = 1 + \frac{M}{K_{m,r,1}} + \frac{S}{K_{2,r,1}} + \frac{S}{K_{1,r,1}} + \frac{M \cdot S}{\eta_{r,1}K_{m,r,1}K_{1,r,1}} + \frac{M \cdot S}{\eta_{r,1}K_{m,r,1}K_{2,r,1}} + \frac{S^2}{\eta_{r,1}^2 K_{1,r,1}K_{2,r,1}}$$

(2.10)

with $\eta_{r,1}$ as an allosteric interaction coefficient between the binding site of the modifier and those of the signal. A similar equation for $\frac{R_2S_4}{\text{max}(R_2S_4)}$ is obtained. Typically, $\text{max}((z_{r,1})^2)$ is obtained at saturating large values of $S$. However, if the active form is not any of the saturated forms of receptor, e.g. $R_1S_2$ as opposed to $R_2S_4$, then the active concentration may reach its maximum at a sub-saturating concentration of $S$ and the maximum is not reached at $\text{max}((z_{r,1})^2)$; this situation we do not consider.

We investigate signaling bias in Figure 2.5 E, F. In these figures we show that a bias in the active receptor conformations can be introduced by different binding affinities of the different states towards the ligand, as shown in Figure 2.5E. Here, a higher affinity of the conformation to the ligand causes a higher fraction of this ligand-bound state to be populated, thus a bias occurs. Similarly, an allosteric modifier shifts the equilibrium of $R_1S_4$ and $R_2S_4$ due to its allosteric interactions (Figure 2.5F). If there is a positive allosteric interaction between the modifier and ligand’s binding sites on the $R_1$ state, i.e. $\eta_{R_1} < 1$, ligand binding to the $R_1$ conformation is enhanced in the presence of modifier $M$, leading to a bias towards this receptor conformation. The same holds true for $\eta_{R_2}$ and the $R_2$ state. The same observation can be made for the competitive modifier, depicted with the blue lines in the same figure. In this case, the receptor state with lower affinity for the modifier $X$ will have a higher probability to be fully occupied by the ligand, which subsequently results in a higher fraction of this state. Thus, the competitive modifier induces an inverse biasing effect.

**Ligand bias induced modulation of signal sensitivity, affinity, and output amplitude**

In addition to influencing ligand bias, modulators influence the dependency of the fraction of active receptor states, e.g. $R_1S_4$, on the concentration of their signals, i.e. $S$. This dose-response relation of $R_1S_4$ with respect to $S$ can be characterized by three parameters: i. it’s Hill coefficient (sensitivity measure, $n_H$), ii. the signal concentration at half maximal response (affinity measure; EC50), and iii. the maximal level of the response (amplitude measure; $\text{max}(R_1S_4)$). Each of these response properties can be under the influence of a modulator; modulators can change the qualitative properties
of the dose-response curve of an active receptor state with respect to a signal. We illustrate various examples of such modulator influences in Figure 2.6.

Figure 2.6: Effect of the modifiers on response amplitude, EC
50 and Hill coefficient. The allosteric modifier (M), equilibrium modifier (β, γ) and competitive inhibitor (X) were incorporated into the receptor model and their effects on the formation of R1S4 were investigated: for its maximum, EC
50 value and hill coefficient, nH.

A) Dose-response curve of R1S4 for increasing [M]. B) Effect of the allosteric modifier M; M was varied between 0 and 10 and max(R1S4) values were calculated accordingly. Red curve: ηR1 = 0.5, ηR2 = 1.56. Blue curve: ηR1 = 2, ηR2 = 0.64. C) Dose-response curve of R1S4 in absence or presence of the competitive inhibitor, X. Black line: X = 0; Dark blue: X = 1; Blue: X = 5, Light Blue: X = 10. D) Dose-response curve of R1S4 for varying β and γ. E) Dependence of EC
50 values of R1S4 on β and γ. β was varied from 0 – 10, γ is fixed at 10 (blue curve). Red: γ varies from 0 – 10, β = 1. F) The Hill coefficient, nH, dependence on the modifiers β (blue) and γ (red). Parameters are listed in the appendix.

Figure 2.6A illustrates the influence of the allosteric modulator M on the response amplitude of R1S4. For a positive allosteric interaction (ηR1 < 1) an increasing modifier concentrations leads to an increase in max(R1S4). In contrast, a higher modifier concentrations decreases the amplitude if the allosteric interaction is negative (i.e. ηR1 > 1). This dependence of modifier influence on whether the allosteric interaction is positive or negative is also illustrated in Figure 2.6B, where the max(R1S4) is plotted as function of the modifier concentration. A competitive modifier, denoted by X,
shifts the $R_{1}S_{4}$ curve to increase the $EC_{50}$ (Figure 2.6C) while the conformation-equilibrium modifiers $\beta$ and $\gamma$ increase and decrease the receptors affinity to the ligand, respectively (Figure 2.6D,E). These latter modifiers also influence the measure for the receptor sensitivity as quantified by the Hill coefficient (Figure 2.6F).

### 2.3 Discussion

In this paper, we presented a model of GPCR activation by extracellular signals that via allosteric interactions and induction of conformation changes bias the receptor towards conformations that preferentially activate particular downstream proteins. Hereby, tuneable ligand biased signaling is obtained at the level of the receptor. This model contains two main features: first, a GPCR is considered to exist in a ligand-dependent receptor-conformation equilibrium of active and inactive receptor states. Second, the intracellular domain of the receptor exists in an equilibrium of (domain) conformations that differ in their affinity for downstream signaling proteins. We used the concept of allosteric ligand binding developed by Monod, Wyman and Changeux, however, the ideas behind it can easily be adapted to the more complex KNF model and generalization thereof. In addition, we illustrated that the ligand biased signaling of the model involves sensitivity, amplitude and affinity modulation as is found in experiments [126, 129].

Recent studies relate the structural information of receptors to their function with free energy landscapes (reviewed in [152]). One view is that a GPCR in the inactive state visits many different conformations for short times (dynamic disorder) without getting "locked" (i.e. spend a long time) in a few particular states to initiate signaling. This means that the free energy landscape, defined over all possible receptor structures, contains no free energy wells in the inactive state; it is essentially flat. Fluctuations in the protein structure by thermal noise then cause the receptor to diffuse over this free energy landscape and the residence time in active states is short as they do not occur at potential wells. One perspective on the effect of signal binding is that it stabilizes particular active receptor conformations and changes the free energy landscape to one with wells, where each well corresponds to an alternative active signaling states. We described those alternative signaling states as the local conformations of the intracellular domain of the receptor. Both for the active and inactive states of the receptor, we coarse-grained the free energy landscape to one where only a small number of structures can co-exist in a ligand-dependent equilibrium. In other words, even though we did not explicitly consider free-energy landscapes, our model that derive from this perspective.

Our proposal to distinguish between global and local conformations of GPCRs is supported by several experimental studies. For instance, Nygaard et al [153], reported the existence of local micro-switches in the intracellular parts of GPCRs, which co-
Conformation-equilibrium model of GPCRs

occur with global conformational changes of the receptors. This distinction allows for the consideration of multiple different activity states of the intracellular GPCR domain, while the entire receptor occupies its active R state, as suggested by Vardy and Roth [154]. This is also indicated by recent NMR experiments and structural simulations, which point to the existence of multiple (intracellular) conformations of GPCRs [118, 128, 154].

It has been suggested that G-proteins bind asymmetrically to GPCR dimers [137, 155]; thus, conformational and signaling structures of the receptors within a dimer might be asymmetrical. This property would allow for inter-receptor crosstalk and allosteric interactions between binding sites of different monomers. Experimental evidence supports this hypothesis. For example, Vilardaga et al. [138] have shown a direct transinhibition of morphine on α2A-AR conformation and signaling activity in α2A-AR/MOR heterodimers. Additionally, Han et al. [155], and Jastrebska et al. [137] reported asymmetric receptor conformations within dopamine receptor heterodimers and rhodopsin receptors, respectively. Therefore, our assumption that allosteric interactions between binding sites of one monomer and sites of its dimer partner do not occur is likely a simplification. It also has several experimentally testable consequences. Allosteric interactions between the receptor subunits of the dimer would lead to an enhanced cooperativity not reproducible by our model. In such a case, expanding the model to allow for allosteric interactions between the subunits might be required. The reason why we did not include these interactions in the first place is that it would increase the number of parameters of the model and require more experiments for parameter inference. Starting with a simpler model is our preferred strategy, as it is not clear if the additional parameters are indeed needed for the model to describe experimental data adequately. We note that the asymmetric conformations of the dimeric receptors is important especially for heterodimers [138, 155]. For instance, ligand binding to one receptor can positively or negatively regulate the signaling activity of its partner receptor. In the model, we focussed on homodimers. As a result a conformation difference between monomers is likely of less importance.

The advantage of our model for the fitting of experimental data is that it offers more mechanistic insight into conformation equilibria of the receptor and how this is influenced by ligand binding and allosteric interactions. The downside is that more parameters are to be determined, minimally three more (i.e. \( L, \ell_R, \ell_T \)). In order to reliably estimate those from experimental data, more experimental data points and experimental conditions are generally required than what is necessary for the fitting of, for instance, the extended allosteric two state model proposed by Hall [145] to experimental data as was done recently [156]. Popular models that are currently used to quantify effects of allosteric modulators on GPCR signaling are based on the allosteric two state model proposed by Ehler [157] or Hall [145] or variants thereof, e.g. [158]. We derive these models in the Supplementary Information and discuss their differences to our approach. The main difference is that the existing models describe the receptor and receptor-ligand interactions in a phenomenological
manner with focus on characterizing the nature of the (allosteric) ligands as agonists or antagonists. In contrast, our model considers a dimer receptor and allows explicitly for different (global and local) conformations. With this approach, our model is able to give insights into the conformation dynamics and the receptor mechanism for ligand biased signaling.

Summarizing, the model we presented considers an equilibrium of different, active-signaling receptor conformations that is influenced by binding of ligands and modifiers and their allosteric interactions. It can be used to study the effects of modifiers on pharmacologically important characteristics of GPCR signaling, such as the Hill coefficient or $EC_{50}$ values, as well as a tool to study mechanisms leading to biased signaling at the level of the receptor.
2.A Equilibrium binding models: examples and basic terminology

Single site binding

We consider a monomer, \( e \), with a single site that binds a ligand, \( S \), i.e.

\[
\begin{align*}
  e + S &\rightleftharpoons es \\
\end{align*}
\]

This reaction is assumed at thermodynamic equilibrium; hence, then it’s rate, \( v \), equals zero,

\[
\begin{align*}
  v = k^+ \cdot e \cdot s - k^- \cdot es &= k^+ \cdot e \cdot s \left( 1 - \frac{es}{k^+ \cdot e \cdot s} \right) = 0 \\
\end{align*}
\]

Therefore, at equilibrium, when \( v = 0 \), we have the following relationship between the kinetic parameters and the equilibrium concentrations,

\[
\begin{align*}
  \frac{es}{e \cdot s} &= \frac{k^+}{k^-} \equiv \frac{1}{K_D} \\
\end{align*}
\]

We note that "\( \text{def} \)" means here "define". Here we have identified the dissociation constant, \( K_D \) as an equilibrium constant. As any equilibrium constant, the \( K_D \) can be expressed in terms of the standard Gibbs free energies of formation of \( e, s, \) and \( es \), which we will not do here.

The total amount of monomer, \( e_T \), is conserved and equals the sum of the concentrations of its two states,

\[
\begin{align*}
  e_T = e + es &= e + \frac{e \cdot s}{K_D} \Rightarrow \frac{e_T}{e} = 1 + \frac{s}{K_D} \equiv z \\
\end{align*}
\]

The "\( z \)" is identical to the partition function defined in equilibrium statistical thermodynamics. It will turn out to be a key role in the models we derive. The concentration of two states of the monomer can now be obtained from the previous equation and the definition of the dissociation constant,

\[
\begin{align*}
  e &= e_T \frac{1}{K_D + s} \\
  es &= e_T \frac{s}{K_D + s} \\
\end{align*}
\]

This indicates a hyperbolic dependency of the concentration of the bound monomer as function of the ligand concentration. The monomer is for 50 % saturated with the
ligand when \( s = K_D \stackrel{\text{def}}{=} s_{0.5} \). We can also express the fractions of the states,

\[
\frac{e}{e_T} = \frac{1}{K_D + s} = \frac{1}{z} \quad (2.17)
\]

\[
\frac{es}{e_T} = \frac{s}{1 + sK_D} = \frac{s}{K_D z} \quad (2.18)
\]

In the next section, we consider two binding sites. If those are independent – no allosteric interaction occurs – then the partition function for this new system equals \((z)^2\). We will exploit throughout this work the property of partition functions of independent sites to give rise to the partition function for a system composed out of several of those sites.

**Two sites on one monomer with an allosteric interaction**

We consider a monomer, \( r \), with two distinct binding sites; for notational convenience one is denoted on the left hand side of \( r \), i.e. when occupied we have \( sr \), and the other on the right hand side of \( r \), i.e. \( rs \),

\[
r + s \rightleftharpoons sr \quad (2.19)
\]

\[
r + s \rightleftharpoons rs \quad (2.20)
\]

\[
sr + s \rightleftharpoons srs \quad (2.21)
\]

\[
rs + s \rightleftharpoons srs \quad (2.22)
\]

At equilibrium we have the following dissociation constant definitions,

\[
K_1 = \frac{r \cdot s}{sr} \quad (2.23)
\]

\[
K_2 = \frac{r \cdot s}{rs} \quad (2.24)
\]

\[
K_3 = \frac{sr \cdot s}{srs} \quad (2.25)
\]

\( K_4 \) is related to the other dissociation constant because of the detailed balance condition: \( K_1K_2K_3K_4 = 1 \). Again, the total monomer concentration is conserved,

\[
r_T = r + sr + rs + srs = r \left( 1 + \frac{s}{K_1} + \frac{s}{K_2} + \frac{s^2}{\alpha K_1K_2} \right) \quad (2.26)
\]

\[
z_{r,1,2} \stackrel{\text{def}}{=} \frac{r_T}{r}, \text{ the partition function} \quad (2.27)
\]

Here we have written \( K_3 \) as \( \alpha K_2 \) to show that we consider binding to the second site in such a manner that the occupation of the first site can alter the affinity of the
second (and vice versa) via allostery. \( \alpha \) is sometimes called an (allosteric) interaction coefficient. If \( 0 < \alpha < 1 \) then we have positive allostery (sensitisation), if \( \alpha > 1 \) we have negative cooperativity and if \( \alpha = 1 \) the sites are independent. In case of independent binding sites, we have \( \alpha = 1 \),

\[
z_{r,1,2} = \left(1 + \frac{s}{K_1} + \frac{s^2}{K_1 K_2}\right) = \left(1 + \frac{s}{K_1}\right) \left(1 + \frac{s}{K_2}\right) = z_{r,1} z_{r,2}
\] (2.28)

And if \( K_1 = K_2 = K \), the partition function of the monomer with the two sites equals of the product of the individual binding sites (considered in isolation),

\[
z_{r,1,2} = \left(1 + \frac{s}{K}\right)^2 = z^2
\] (2.29)

Again the concentration of the monomer states can be derived from the definitions in this section,

\[
\begin{align*}
r &= r_T \frac{1}{z_{r,1,2}} \\
sr &= r_T \frac{s}{K_1} \frac{1}{z_{r,1,2}} \\
r_s &= r_T \frac{s}{K_2} \frac{1}{z_{r,1,2}} \\
srs &= r_T \frac{\alpha s^2}{K_1 K_2} \frac{1}{z_{r,1,2}}
\end{align*}
\] (2.30) (2.31) (2.32) (2.33)

In the next section, we derive an example of a GPCR model and we will show that the monomer partition function, even in the case of multiple binding per monomer, e.g. \( z_{r,1,2} \), occurs as independent terms in the GPCR model as long as the monomers act independently. Then, the GPCR model can be derived as function of the partition function of the monomers; hence, the monomers act as proper modules in this case.

**Example GPCR model**

*Without modifiers affecting the conformation equilibria*

We consider a dimer with two identical monomers that each have two different binding sites for the ligand \( S \). The monomers within the dimer can be in two global conformations, denoted by \( R \) and \( T \); hence, we can have \( RR, RT, TR, \) and \( TT \) as receptor conformation states. The Monod-Wyman-Changeux (MWC) model [139] assumes that the \( RT \) and \( TR \) states do not occur due to strong cooperative interactions between the monomers, called "concerted symmetry". In addition, we assume that each monomer can have two local domain conformations of their intracellular binding
domains regardless of their global state. We denote the local states as a subscript to the global state, i.e. we have $R_1, R_2, T_1,$ and $T_2$. We also assume concerted symmetry for local conformations, i.e. $R_1 R_2$ and $T_1 T_2$, do not occur, only $R_1 R_1$, $R_2 R_2$, $T_1 T_1$ and $T_2 T_2$. For brevity we denote those dimers by $R_1$, $R_2$, $T_1$, and $T_2$. The total amount of receptor dimer $R_T$ is now the sum of the concentrations of all the states,

$$R_T = R_1 + 4R_1 S + 6R_1 S_2 + 4R_1 S_3 + R_1 S_4$$
$$+ R_2 + 4R_2 S + 6R_2 S_2 + 4R_2 S_3 + R_2 S_4$$
$$+ T_1 + 4T_1 S + 6T_1 S_2 + 4T_1 S_3 + T_1 S_4$$
$$+ T_2 + 4T_2 S + 6T_2 S_2 + 4T_2 S_3 + T_2 S_4$$

(2.34)

Thus we have 4 forms of the states with 1 ligand bound to the dimer, 6 with 2, 4 with 3, 1 with 0, and 1 with 4. If the monomers act independently then the partition functions of the dimer states are the product of the monomer states. Then, we arrive at,

$$R_T = R_1 z_{R,1} + R_2 z_{R,2} + T_1 z_{T,1} + T_2 z_{T,2}$$
$$= R_1 (z_{r,1})^2 + R_2 (z_{r,2})^2 + T_1 (z_{t,1})^2 + T_2 (z_{t,2})^2$$
$$= R_1 \left(\left((z_{r,1})^2 + \frac{R_2}{R_1} (z_{r,2})^2\right) + \frac{T_1}{R_1} \left((z_{t,1})^2 + \frac{T_2}{T_1} (z_{t,2})^2\right)\right)$$
$$\overset{\text{def}}{=} R_1 \left(\left((z_{r,1})^2 + \ell_R (z_{r,2})^2\right) + L \left((z_{t,1})^2 + \ell_T (z_{t,2})^2\right)\right)$$

(2.35)

The partition function of the entire receptor system equals $R_T / R_1$. The dimer partition functions are now defined as $z_{R,1} = \frac{R_1 T}{R_1} = (z_{r,1})^2$, $z_{R,2} = \frac{R_2 T}{R_2} = (z_{r,2})^2$, $z_{T,1} = \frac{T_1 T}{T_1} = (z_{t,1})^2$, and $z_{T,2} = \frac{T_2 T}{T_2} = (z_{t,2})^2$. (Here the subscript "T" denotes a total concentration, corresponding to the four lines in equation 2.34.) With $z_{r,1}$, $z_{r,2}$, $z_{t,1}$, and $z_{t,2}$ as the monomer partition function given their intracellular domain binding state; those could for instance be,

$$z_{i,j} = 1 + \frac{s}{K_{1,i,j}} + \frac{s}{K_{2,i,j}} + \frac{s^2}{\alpha_{i,j} K_{1,i,j} K_{2,i,j}}$$

(2.36)

with $i = \{r, t\}$ and $j = \{1, 2\}$. If for instance a modulator $m$ can bind in addition to the ligand $s$ and in an independent manner then the partition function of the monomer would become,

$$z_{i,j} = \left(1 + \frac{m}{K_{M,i,j}}\right) \left(1 + \frac{s}{K_{1,i,j}} + \frac{s}{K_{2,i,j}} + \frac{s^2}{\alpha_{i,j} K_{1,i,j} K_{2,i,j}}\right)$$

(2.37)

with $i = \{r, t\}$ and $j = \{1, 2\}$. The $1 + \frac{m}{K_{M,i,j}}$ term is the partition function for the modulator binding site on the monomer.
**Derivation of the allosteric coefficient \( \alpha \)**

The allosteric coefficient \( \alpha \) introduced in our partition functions describes the occupancy of one site on the ligand affinity of the other site. Thus, \( \alpha \) describes the allosteric interactions between binding sites on a monomer. We shall now derive the \( \alpha \) coefficient from a MWC approach to show that \( \alpha \) can be viewed as deriving from the occurrence of different monomer conformations. So, suppose that a monomer \( m \) is in equilibrium between two different conformations, denoted by \( x \) and \( y \) in the following text,

\[
m_x \rightleftharpoons m_y. \tag{2.38}
\]

Then, if the binding of ligands stabilizes one of these conformations (\( x \) or \( y \)), the equilibrium is shifted towards this state, the classical MWC model. Here, the ratio \( m_y / m_x = \mathcal{L} \) describes the equivalent of a \( L \) coefficient in the MWC model. The total concentration of monomer, \( m_T \), equals the sum of the concentrations of all its states:

\[
m_T = m_x + m_x s_1 + m_x s_2 + m_x s_1 s_2 + m_y + m_y s_1 + m_y s_2 + m_y s_1 s_2
\]

\[
= m_x + \frac{m_x s_1}{K_{1,x}} + \frac{m_x s_2}{K_{2,x}} + \frac{m_x s_1 s_2}{K_{1,x} K_{2,x}} + m_y + \frac{m_y s_1}{K_{1,y}} + \frac{m_y s_2}{K_{2,y}} + \frac{m_y s_1 s_2}{K_{1,y} K_{2,y}}
\]

\[
= (m_x + m_y) \left( \frac{m_x s_1}{m_x + m_y K_{1,x}} + \frac{m_x s_2}{m_x + m_y K_{2,x}} + \frac{m_x s_1 s_2}{m_x + m_y K_{1,x} K_{2,x}} + \frac{m_y s_1}{m_x + m_y K_{1,y}} + \frac{m_y s_2}{m_x + m_y K_{2,y}} + \frac{m_y s_1 s_2}{m_x + m_y K_{1,y} K_{2,y}} \right) \tag{2.39}
\]

The partition function for the monomer that results from this approach is rather complicated. In order to avoid this, we express the partition function of the monomer in a more simple format: a so-called Adair model. To achieve this, it is easier to work with conformation fractions, such as \( m_y / (m_x + m_y) \), than with the \( \mathcal{L} \). Let us now
derive the partition function of the monomer, \( z_m \), expressed in the Adair formalism,

\[
\frac{m_{\text{total}}}{m_x + m_y} \overset{\text{def}}{=} z_m = 1 + s_1 \left( \frac{m_x}{m_x + m_y \frac{1}{K_{1,x}}} + \frac{m_y}{m_x + m_y \frac{1}{K_{1,y}}} \right) \\
+ s_2 \left( \frac{m_x}{m_x + m_y \frac{1}{K_{2,x}}} + \frac{m_y}{m_x + m_y \frac{1}{K_{2,y}}} \right) \\
+ s_1 s_2 \left( \frac{m_x}{m_x + m_y \frac{1}{K_{1,x} K_{2,x}}} + \frac{m_y}{m_x + m_y \frac{1}{K_{1,y} K_{2,y}}} \right)
\]

\[
= 1 + \frac{s_1}{K_{1}^*} + \frac{s_2}{K_{2}^*} + \frac{s_1 s_2}{(K_{1} K_{2})^*} \\
= 1 + \frac{s_1}{K_{1}^*} + \frac{s_2}{K_{2}^*} + \frac{s_1 s_2}{\alpha K_{1}^* K_{2}^*} \Rightarrow \text{Adair model (2.40)}
\]

Here, the \( K^* \)'s are the average dissociation constants, considering the fractions of the monomer in \( x \) and \( y \) state. The Adair model (equation 2.40) describes the partition function of the monomer in a concise manner that is less complicated than the underlying MWC model it derives from. An important assumption is that \( \mathcal{L} \) to remain fixed. Summarising, the \( \alpha \) coefficient is defined as an allosteric in interaction coefficient deriving from a conformation transition at the monomer level. The use of Adair model simplifies the structure of the partition function for the whole receptor (equation 2.43).

Next, we use the partition function of the monomer to derive the partition function for the dimeric receptor. We have to take into account that the monomers can in the dimer engage in new conformational changes that lead to the formation of \( R \) and \( T \) monomer states. As we assume concerted symmetry for the receptor, the partition function of the dimer becomes,

\[
Z = (z_r)^2 + L(z_t)^2
\]

and additionally including the (local) domain states for the receptor, we end up with the \( Z \) function mentioned in the main text,

\[
Z = (z_{r,1})^2 + \ell_r (z_{r,2})^2 + L((z_{t,1})^2 + \ell_t (z_{t,2})^2).
\]

In order to show that this description does not violate thermodynamic equilibrium, we have to show that this model obeys: i) detailed balance and ii) mass conservation.
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- Detailed balance: Let us now have a closer look on the state transitions in the full model, now also taking the $x$ and $y$ states into account (Fig. 2.7). Hereby, we also assume concerted symmetry, so the monomers in a dimer will both be either in $x$ conformation or both in $y$, described by $\mathcal{R}_x$ and $\mathcal{R}_y$. The transitions are now: $\mathcal{R}_x \xrightarrow{\mathcal{L}} \mathcal{R}_y \xrightarrow{\frac{1}{\mathcal{L}}} \mathcal{T}_y \xrightarrow{\frac{1}{\mathcal{L}}} \mathcal{R}_x$. The products of the equilibrium constants cancel out to one: $\mathcal{L} \cdot \frac{1}{\mathcal{L}} \cdot \frac{1}{\mathcal{L}} \cdot L = 1$. Thus, detailed balance is not violated.

- Conservation of mass: We derive the partition functions while we sum over all the concentrations of the receptor states and assume all binding reactions at equilibrium. Hence, we account for all mass in the system and mass conservation is not violated.

Figure 2.7: Equilibrium transitions including additional conformational states. $\mathcal{R}_i$ and $\mathcal{T}_i$ representing the different conformations of the receptor dimer. We assume concerted symmetry within the dimer monomers to reduce the complexity of the scheme. This does not influence the outcome if $\mathcal{L}$ is fixed.

Incorporating $L$ and $\ell$ modulators

In equation 2.37, we added a modulator binding site to each monomer, with a monomer binding state dependent affinity. It is a custom in MWC models to include modulators – ligands that do not give rise to protein activity themselves – in the equilibrium constants for conformations, i.e. in $\ell$’s and $L$. This is straightforward and just means that in equation 2.35 not $\mathcal{R}_1$, $\mathcal{R}_2$, $\mathcal{T}_1$, and $\mathcal{T}_2$ are removed out of the brackets but those concentrations multiplied with the partition function of the modulator binding site, e.g. $\mathcal{R}_1 \left(1 + \frac{m}{K_{M,i,j}}\right)^2$. This means that the definitions of the equilibrium
constant for the conformations become,

\[ L = L' \left( 1 + \frac{m}{K_{M,r,1}} \right)^2 \left( 1 + \frac{m}{K_{M,t,1}} \right)^2 \]

\[ \ell_R = \ell'_R \left( 1 + \frac{m}{K_{M,r,2}} \right)^2 \left( 1 + \frac{m}{K_{M,t,1}} \right)^2 \]

\[ \ell_T = \ell'_T \left( 1 + \frac{m}{K_{M,t,2}} \right)^2 \left( 1 + \frac{m}{K_{M,t,1}} \right)^2 \] (2.44)

Also in more complicated cases, with additional modulators, the partition functions will always appear in the numerator and denominator terms.

We note that if the modulators display allosteric interactions with signaling ligands then the monomer partition functions cannot be written as product of modular and signaling ligands partition functions – as they do not function independently – and, as a result, the modulators cannot be subsumed in the definitions of \( \ell \) and \( L \). This indicates the somewhat artificial distinction in MWC models between modulators and signaling ligands.

**The signaling state of the receptor**

Say that \( R_1S_4 \) is one of the signaling states of the receptor and we would like to know how this active conformation changes as function of the concentration of the ligand, \( S \), then we first need to express the fraction of this state. We would obtain,

\[ \frac{R_1S_4}{R_T} = \frac{s^2}{(z_{r,1})^2 + \ell_R (z_{r,2})^2 + L (z_{t,1})^2 + \ell_T (z_{t,2})^2} \] (2.45)

The denominator is the partition function of the receptor expressed in terms of the partition functions of its monomer given their states.

**Biased signaling**

The concentration of \( R_2S_4 \) equals,

\[ \frac{R_2S_4}{R_T} = \frac{\ell' \left( \frac{s^2}{\alpha_{r,2}K_{R_2,1}K_{R_2,2}} \right)^2}{(z_{r,1})^2 + \ell_R (z_{r,2})^2 + L (z_{t,1})^2 + \ell_T (z_{t,2})^2} \] (2.46)
Hence, the ratio of two local conformation of the active \( R \) state equals,

\[
\frac{R_1 S_4}{R_2 S_4} = \frac{\left( \frac{s^2}{\alpha_{r,1} K_{R,1,1} K_{R,1,2}} \right)^2}{\ell_R \left( \frac{s^2}{\alpha_{r,2} K_{R,2,1} K_{R,2,2}} \right)^2} = \frac{1}{\ell_R} \frac{\left( \frac{\alpha_{r,2} K_{R,2,1} K_{R,2,2}}{\alpha_{r,1} K_{R,1,1} K_{R,1,2}} \right)^2}{(z_{r,1})^2}
\]

This is a constant if no ligand bias occurs; then, we require that no modifier or other signal influences \( \ell_R \) and/or \( K_{R,i,j} \) (with \( i, j = \{1, 2\} \)). In the main text, we consider the situation where the second factor equals 1 and ligand bias can only be induced via an influence on \( \ell_R \).

**A note on the bias plots**

In a bias plot, the normalised signaling response of one route, i.e. \( \frac{R_2 S_4}{\max(R_2 S_4)} \), is plotted as function of the normalised response of an alternative route, \( \frac{R_1 S_4}{\max(R_1 S_4)} \). These ratios are given by the following equations,

\[
\frac{R_1 S_4}{\max(R_1 S_4)} = \frac{\left( \frac{s^2}{\alpha_{r,1} K_{1,r,1} K_{2,r,1}} \right)^2}{\left( 1 + \frac{s}{K_{1,r,1}} + \frac{s}{K_{2,r,1}} + \frac{s^2}{\alpha_{r,1} K_{1,r,1} K_{2,r,1}} \right)^2} = \frac{(z_{r,1})^2}{Z}
\]

And the same for \( R_2 S_4 \) and \( \max(R_2 S_4) \); note that in this case both terms involve a multiplication with \( \ell_R \) and that therefore \( \ell_R \) drops out of the equation. As result, ligand bias only occurs if the dissociation constants, the \( K \)'s, are influenced by modulators via competitive binding or via allosteric interactions between modulator and signal binding sites. We also note that if a modifier is present the partition function of the monomers obtain a modifier concentration dependent term (examples can be found in the box in the main text). Then, multiple active forms could also play role, e.g. \( R_1 S_4 M \) in addition to \( R_1 S_4 \) (with \( R_1 S_4 M \) denoting a modifier bound \( R_1 S_4 \) state). For instance, one could have,

\[
\frac{R_1 S_4 + R_1 S_4 M}{\max(R_1 S_4 + R_1 S_4 M)} = \frac{\left( \alpha_{r,1} K_{1,r,1} K_{2,r,1} K_{m,r,1} + \frac{s^2}{\alpha_{r,1} K_{1,r,1} K_{2,r,1}} \right)^2}{\max((z_{r,1})^2)}
\]

\[
z_{r,1} = 1 + \frac{m}{K_{m,r,1}} + \frac{s}{K_{1,r,1}} + \frac{s}{K_{2,r,1}} + \frac{ms}{\eta_{r,1} K_{m,r,1} K_{1,r,1}} + \frac{ms}{\eta_{r,1} K_{m,r,1} K_{2,r,1}} + \frac{s^2}{\alpha_{r,1} K_{1,r,1} K_{2,r,1}} + \frac{ms^2}{\eta_{r,1} K_{1,r,1} K_{2,r,1}}
\]

(2.49)
2.B Hill coefficient

One manner to characterise the sensitivity of a cooperative receptor system with allosteric interactions between ligands and modulators is to determine its Hill coefficient with respect to a particular modulator or signaling ligand. Thus, the receptor is viewed with respect to one concentration, $s$, i.e. the modulator or ligand of interest, and this function is approximated by a Hill equation,

$$y = y_T \frac{s^n}{K^n + s^n}$$

(2.50)

Here $y$ is an output of the signaling receptor, e.g. downstream signaling response or the concentration of a particular receptor state (e.g. $R_1 S_4$). From this equation the Hill coefficient $n$ can be determined from,

$$n = 2 \left. \frac{\partial \ln y}{\partial \ln s} \right|_{s=s_{0.5}}$$

(2.51)

Here $s_{0.5}$ denotes the concentration of $s$ where $y = \frac{y_T}{2}$ given a value for $K$ and $n$.

2.C Deriving GPCR models

Through the decades, a plethora of concepts have been developed to describe cooperativity and allosteric ligand binding in oligomeric proteins. These models differ in their complexity as well as in the underlying purpose they have been developed for. Our model includes the dimeric nature of membrane receptors, as well as different conformations of the receptor’s domains ($R_1, R_2$). Additionally, we consider alternative binding sites for the orthosteric ligand and the allosteric modifiers. The two-state model derived in section 2.C in this Appendix describes the basic assumptions underlying our model, whereas the allosteric two-state model proposed by Hall [145], derived in section 2.C slightly differs in its concept. The $\alpha$-coefficient in our model describes an allosteric interaction coefficient and corresponds to $\gamma$ in the Hall model, whereas $\alpha, \beta$ and $\delta$ describe changes in ligand affinity to the receptor. Those are described in our model by different $K_D$ values.

The two-state model of receptor activation

The two-state model of receptor activation as described by Paul Leff in 1995 [159] is closely related to the MWC model and describes the very basic concepts of the receptor model introduced in our article. Notably, it does not consider domain conformations. The two-state model assumes a single ligand to bind to an active or inactive receptor state with different affinities, thereby also shifting the equilibrium
between the receptor states \((L)\). In MWC terminology, the total receptor can be written as,

\[
R_T = R + RS + T + TS
= R \left( 1 + \frac{s}{K_R} \right) + LR \left( 1 + \frac{s}{K_T} \right)
= R \left( \left( 1 + \frac{s}{K_R} \right) + L \left( 1 + \frac{s}{K_T} \right) \right)
\] (2.52)

Consequently, the partition function simplifies to

\[
z = \left( 1 + \frac{s}{K_R} \right) + L \left( 1 + \frac{s}{K_T} \right)
\] (2.53)

### The allosteric two-state model

The two-state model derived in the previous section has been extended by David Hall [145] in order to include the effects of allosteric ligands with a focus on the characterization of pharmacological ligands. Here, two ligands of different identity are assumed to bind the receptor states with varying affinities, thereby influencing both the binding affinity of the other ligand as well as the equilibrium between inactive and active receptor states.

Hence, the total receptor amount is determined by the inactive and active receptor states bound to the ligands A and B, and their combination, respectively. Written in MWC terminology, this leads to

\[
R_T = R + RA + RB + RAB + T + TA + TB + TAB
= R \left( 1 + \frac{\alpha A}{K_A} + \frac{\beta B}{K_B} + \frac{\alpha \beta \gamma \delta AB}{K_A K_B} \right)
+ L \left( 1 + \frac{A}{K_A} + \frac{B}{K_B} + \frac{\gamma AB}{K_A K_B} \right)
\] (2.54)

and

\[
z = 1 + \frac{\alpha A}{K_A} + \frac{\beta B}{K_B} + \frac{\alpha \beta \gamma \delta AB}{K_A K_B}
+ L \left( 1 + \frac{A}{K_A} + \frac{B}{K_B} + \frac{\gamma AB}{K_A K_B} \right)
\] (2.55)

with \(\alpha\) and \(\beta\) being the affinity of ligand A and B for R and T, respectively, while \(\gamma\) corresponds to the allosteric interaction coefficient of A and B. \(\delta\) represents the
"allosteric activation interaction", the affinity of one ligand to the active state when the other ligand is already bound, thus, whether it stabilizes the active receptor state or not. Please note, that the definitions for $\alpha$, $\beta$, $\gamma$ and $\delta$ are adopted from the original paper, whereas the $L$ coefficient is defined here as $\frac{T}{R}$ while in the original text it is the opposite. For more detailed explanation, see [145].

**General approach**

In section 2.A we have derived the partition function for a dimeric GPCR with two ligand binding sites per monomer, and have seen that in this case, the partition function of one specific conformation equals $(z_{i,j})^2$, with $i = \{r, t\}$ and $j = \{1, 2\}$. Subsequently, a partition function of a specific state of a multi-meric GPCR equals $(z_{i,j})^m$, with $i = \{r, t\}$ and $j = \{1, 2\}$ and $m = \text{the number of monomers the receptor is composed of}$. The derivation of the partition function can thus be generalized by making use of the binomial theorem:

$$(1 + x)^n = \sum_{k=0}^{n} \binom{n}{k} x^k. \quad (2.56)$$

Here, $n$ is denoting the amount of ligand binding sites in the protein, $k$ denotes the occupancy status per subunit (ligand bound or not).

Thus, $z_{i,j}$ for a dimer with two distinct ligand binding sites per subunit becomes:

$$z_{i,j} = \left(1 + \frac{s}{K_{1,i,j}} + \frac{s}{K_{2,i,j}} + \frac{s^2}{K_{1,i,j}K_{2,i,j}}\right)^2 \quad (2.57)$$

$$= \left(\sum_{k=0}^{1} \sum_{l=0}^{1} \binom{1}{k} \binom{1}{l} \left(\frac{s}{K_{1,i,j}}\right)^k \left(\frac{s}{K_{2,i,j}}\right)^l\right)^2 \quad (2.58)$$

with $i = \{r, t\}$ and $j = \{1, 2\}$, and $k$ and $l$ denoting the occupancy status of the individual binding sites where the dissociation constant is, say, $K_{1,i,j}$ and $K_{2,i,j}$, respectively. In the same way, a general partition function can be generated:

$$z_{i,j} = \left(\sum_{k=0}^{f} \sum_{y=0}^{w} \binom{f}{k} \binom{w}{y} \left(\frac{s}{K_{1,i,j}}\right)^k \left(\frac{s}{K_{x,i,j}}\right)^y\right)^n \quad (2.59)$$

with $i = \{r, t\}$ and $j = \{1, 2\}$, $x = \text{number of different binding sites}$, $f, ..., w = \text{the number of identical binding sites (i.e. with the same } K_D \text{) per monomer}$, $k, ..., y$ the occupation status of identical binding site $f, ..., w$, and $n = \text{number of monomers}$. This approach is only feasible if there are no allosteric interactions between the ligand binding sites.
2. D Model parameters

For the figures, different parameter values were chosen. In the following section, the parameter values are listed that resulted in the figures shown in the main text.

Figure 2.4: Conformation changes of active states as function of signal and modifier concentrations.

Fig. 2.4A) $K_{1,R1} = K_{2,R1} = 1,$  
$\alpha_{R1} = \alpha_{R2} = 0.1,$  
$K_{1,R2} = K_{2,R2} = 5,$  
$K_{1,T1} = K_{2,T1} = 15,$  
$\alpha_{T1} = \alpha_{T2} = 1,$  
$K_{1,T2} = K_{2,T2} = 25,$  
$\ell_T = \ell_R = 1,$  
$L = 50, X = 0, M = 0;  

Fig. 2.4B) solid blue line: $L = 50,$ purple dashed line: $L = 1; \text{remaining parameters: see Fig.2.4A).}$

Fig. 2.4C) see Fig.2.4A)

Fig. 2.4D) see Fig.2.4A)

Fig. 2.4E) see Fig.2.4A)

Fig. 2.4F)$\ell_R = (1 + \alpha)^2, \alpha = 100; \text{remaining parameters: see Fig.2.4A).}$

Figure 2.5: Quantification of ligand bias.

Fig. 2.5C)$K_{1,R1} = K_{2,R1} = 0.85,$  
$K_{1,R2} = K_{2,R2} = 1,$  
$K_{1,T1} = K_{2,T1} = 10,$  
$K_{1,T2} = K_{2,T2} = 10,$  
$\alpha_{R1} = \alpha_{R2} = 0.1,$  
$\alpha_{T1} = \alpha_{T2} = 1,$  
$\ell_T = 10, \ell_R = 1,$  
$L = 10, X = 0, M = 0;  

Fig. 2.5D) K_{1,R1} = K_{2,R1} = 1,$  
$K_{1,R2} = K_{2,R2} = 1,$  
$\ell_R = \left(\frac{1 + \beta_R}{1 + \gamma_R}\right)^2,$  
solid black line: $\beta = 0, \gamma = 0; \text{dashed line: } \beta = 1, \gamma = 0; \text{dotted line: } \beta = 0, \gamma = 1; \text{remaining parameters: see Fig.2.5C).}$
Fig. 2.5E) black, solid line: $K_{1,R1}=K_{2,R1}=1, K_{1,R2}=K_{2,R2}=1$; dashed line: $K_{1,R1}=K_{2,R1}=0.3, K_{1,R2}=K_{2,R2}=1$; dotted line: $K_{1,R1}=K_{2,R1}=3, K_{1,R2}=K_{2,R2}=1$; remaining parameters: see Fig.2.5C.

Fig. 2.5F) black solid line: $X=0, M=0$; red, dashed line: $X=0, M=100, K_M=1, \eta_{R1}=0.1, \eta_{T1}=\eta_{T2}=1$; red, dotted line: $X=0, M=100, K_M=1, \eta_{R1}=1, \eta_{T1}=\eta_{T2}=1$; blue, dashed line: $X=3, M=0, K_{X,R1}=1, K_{X,R2}=0.1, K_{X,T1}=K_{X,T1}=1$; blue, dotted line: $X=3, M=0, K_{X,R1}=0.1, K_{X,R2}=1, K_{X,T1}=K_{X,T1}=1$; remaining parameters: see Fig.2.5C.

Figure 2.6: Effect of the modifiers on response amplitude, EC$_{50}$ and Hill coefficient.

Fig. 2.6A) $K_{1,R1}=K_{2,R1}=1$,
$K_{1,R2}=K_{2,R2}=1$,
$K_{1,T1}=K_{2,T1}=10$,
$K_{1,T2}=K_{2,T2}=10$,
$\alpha_{R1}=\alpha_{R2}=0.1$,
$\alpha_{T1}=\alpha_{T2}=1$,
$L_T=10$, $L_R=1$,
$L=10, X=0$;
black line: $M=0$; dark blue line: $M=0.1, \eta_{R1}=5, \eta_{R2}=0.5$;
blue line: $M=1, \eta_{R1}=5, \eta_{R2}=0.5$;
light blue line: $M=10, \eta_{R1}=5, \eta_{R2}=0.5$;
dark red line: $M=0.1, \eta_{R1}=0.5, \eta_{R2}=5$;
red line: $M=1, \eta_{R1}=0.5, \eta_{R2}=5$;
light red line: $M=10, \eta_{R1}=0.5, \eta_{R2}=5$;

Fig. 2.6B) $K_{1,R1}=K_{2,R1}=0.3$,
$K_{1,R2}=K_{2,R2}=1$,
$K_{1,T1}=K_{2,T1}=10$,
$K_{1,T2}=K_{2,T2}=10$,
$\alpha_{R1}=\alpha_{R2}=1$,
$\alpha_{T1}=\alpha_{T2}=1$,
$L_T=10$, $L_R=1$,
$L=1000 \left( \frac{1+\beta}{1+\gamma} \right)^2, X=0, M=0$; black line: $\beta=50, \gamma=20$;
dark blue line: $\beta=60, \gamma=20$;
blue line: $\beta=75, \gamma=20$;
light blue line: $\beta=90, \gamma=20$;
dark red line: $\beta=50, \gamma=25$;
red line: $\beta=50, \gamma=35$;
light red line: $\beta=50, \gamma=45$
**Fig. 2.6C)** $L = 10$; blue line: $\eta_{R1} = 2$, $\eta_{R2} = 0.64$; red line: $\eta_{R1} = 0.5$, $\eta_{R2} = 1.56$; remaining parameter: see Fig. 2.6A).

**Fig. 2.6D)** $K_{1,R1} = K_{2,R1} = 0.3$,
$K_{1,R2} = K_{2,R2} = 1$,
$K_{1,T1} = K_{2,T1} = 10$,
$K_{1,T2} = K_{2,T2} = 10$,
$\alpha_{R1} = \alpha_{R2} = 1$,
$\alpha_{T1} = \alpha_{T2} = 1$,
$\ell_T = 10$, $\ell_R = 2$,
$L = 100 \left( \frac{1+\beta}{1+\gamma} \right)^2$, $X=0$, $M=0$; blue line: $\beta$ is varied from 0 to 10, $\gamma = 10$; red line: $\beta = 1$, $\gamma$ varies from 0 to 10.

**Fig. 2.6E)** black, solid line: $X=0$, dark blue line: $X=1$, blue line: $X=2.5$, light blue line: $X=5$; $K_{X,R1} = K_{X,R2} = 1$, $\alpha_{R1} = \alpha_{R2} = 0.88$, $L = 1000$; $\ell_R = 1$, remaining parameter: see Fig. 2.6B).

**Fig. 2.6F)** $K_{1,R1} = K_{2,R1} = 0.3$,
$K_{1,R2} = K_{2,R2} = 1$,
$K_{1,T1} = K_{2,T1} = 10$,
$K_{1,T2} = K_{2,T2} = 10$,
$\alpha_{R1} = \alpha_{R2} = 0.8$,
$\alpha_{T1} = \alpha_{T2} = 1$,
$\ell_T = 10$, $\ell_R = 1$,
$L = 10 \left( \frac{1+\beta}{1+\gamma} \right)^2$, $X = 0$, $M = 0$;
blue line: $\beta$ varied between 0 and 10, $\gamma = 1.4$;
red line: $\beta = 3.7$, $\gamma$ varied between 0 and 10.
Biased activation of $G_{aq}$ by calcium-sensing receptor agonists
Abstract

**Background** G protein-coupled receptors (GPCRs) generally activate multiple downstream signaling pathways, via different G proteins. Which G protein is activated, and to what extent, depends on which ligands are bound to the receptor. Ligand combinations stabilize receptor conformations with particular downstream signaling biases. Signaling bias is typically inferred from activation of downstream signaling read-outs. It is therefore not always clear whether bias is caused by the GPCR or by downstream processes.

**Aim** Here we study the ligand bias of the extracellular calcium sensing receptor (CaSR). We measure direct activation of the G protein $G_q$ in response to several direct activators of CaSR, allosteric modulators and drugs.

**Results** Using Förster Resonance Energy Transfer (FRET), we monitored the real-time activation of $G_{aq}$ by CaSR in single cells. We characterized steady-state signaling, for all considered ligand combinations, in terms of half-maximal activation ($EC_{50}$), signal sensitivity (the Hill coefficient) and maximal activation, to assess ligand-ligand interactions leading to biased signaling by the CaSR.

**Conclusion** Particular ligand combinations induced biased activation of $G_{aq}$. Other ligand combinations, which were earlier found to give biased activation of downstream signaling, did not show biased activation of the $G_{aq}$ protein, suggesting that, in those cases, signaling bias does not occur at the CaSR but rather downstream. Biased activation of outputs of GPCR-networks is therefore caused by concerted bias of the GPCR and the downstream signaling network.

### 3.1 Introduction

G protein-coupled receptors (GPCRs) are cell-surface receptors that connect the signaling machinery of a cell to its extracellular environment. GPCRs are one of the largest receptor families encoded on the human genome [6] and are involved in a broad range of different functions. Due to their versatility, they are interesting drug targets and currently about 30 - 40% of marketed drugs are acting on GPCRs [160, 161].

Once activated, the receptor binds and activates different intracellular signaling proteins, e.g. heterotrimeric G proteins and β-arrestins, presumably via dedicated receptor conformations [162], reviewed in [161]. In the inactive state, $G_\alpha$ subunits are bound to GDP and are coupled to the $G_{\beta\gamma}$ subunit. Binding to an active receptor induces conformational changes within the G protein, leading to a GDP release and the binding of GTP in the $\alpha$ subunit. Subsequently, $G_\alpha$ and $G_{\beta\gamma}$ subunits dissociate or change conformation and activate downstream signaling proteins. A single receptor can therefore activate multiple intracellular proteins.

Extracellular ligands can introduce biases in intracellular signaling, by stabilizing
Biased activation of \( G_{aq} \) by the CaSR

distinct receptor conformations that each activate downstream signaling pathways to different degrees [69]. Understanding how ligand-ligand interactions at the level of a GPCR brings about biased signaling has been recognized as a major challenge in the search for ‘biasing drugs’ [81–83]. Ligand bias, or biased agonism, has been documented for several GPCRs ([70, 74–77]). In most studies, the biasing effects of ligands is deduced from shifts in dose-response curves of downstream signaling responses [80, 93, 94]. Agonist screenings have revealed the biasing effects of clinically relevant compounds [84, 85], and pharmacologic active compounds with modulated biasing effects were developed [86, 87]. However, different sources of bias have been distinguished, ranging from receptor to network contributions [80, 163]. Even though theory has been used to relate agonist bias to the free energy landscape of a receptor [88, 89], few experimental studies focus on ligand bias at the level of a GPCR. Most studies focus on bias observed at output of the signaling network, downstream of GPCRs. Therefore, whether bias is induced at the receptor level or within the signaling network is often unknown.

In this work, we use the human extracellular calcium sensing receptor (CaSR), involved in whole-body calcium homeostasis [50, 54]. In addition to calcium, it binds several other ligands, such as di- and trivalent ions, amino acids, polyamines and antibiotics [55]. It has recently been characterized as an amino-acid sensor [164, 165]. Mutations in this receptor are associated with several diseases, most noticeably familial hypocalciuric hypercalemia (FHH) and autosomal dominant hypocalcemia (ADH), induced by a gain-of-function mutation of the CaSR [67, 166].

The CaSR couples to three classes of heterotrimeric \( G_\alpha \) subunits (\( G_{aq} \), \( G_i \) and \( G_{12/13} \)) and to \( \beta \)-arrestins to activate several downstream signaling pathways [55]. The biasing influence of several CaSR ligands on signaling-pathway activation has been investigated using downstream signaling read-outs such as intracellular calcium release, ERK phosphorylation, membrane ruffling, IP\(_1\) accumulation and decrease in cAMP concentrations [66, 78, 167, 168]. Despite the great interest in ligand-biased signaling by GPCRs, remarkably few studies focus at the signaling bias introduced by the GPCR itself. Most studies focus on biases observed at downstream signaling readouts. It remains therefore unclear to what extent these biases can be attributed to a GPCR, to its downstream signaling network, or a combination of both. One would expect that part of the tasks of the cellular signaling network is to integrate signals and infer the appropriate biases, suggesting that the role of GPCRs are perhaps only minor.

Here we addressed the contribution of the CaSR to ligand-biased signaling. We measured real-time, single-cell responses of CaSR-induced \( G_{aq} \) activation to different ligand combinations, using a \( G_q \) FRET sensor.
3.2 Results

**G_q** activation as a direct measure of CaSR induced ligand bias

We focus on CaSR-mediated **G_q** activation to address ligand bias introduced at the level of the GPCR. HEK293 cells, stably expressing the CaSR (CaSR-HEK cells), were transfected with a FRET sensor for **G_q** activation, described previously [169]. This sensor is a loss-of-FRET sensor; implying that a drop in signal represents an increase of **G_q** activity. The fluorescence at different emission wavelengths of the **G_q** FRET construct were recorded during receptor stimulation and their ratio (YFP/CFP) was used to determine the CaSR-induced **G_q** activation (Fig. 3.1A). This FRET construct gives a ratiometric output and therefore corrects for the sensor expression differences and other sources of concentration fluctuations.

**Figure 3.1: The FRET reporter and the CaSR modulators, used in this study.** A) Working principle of the **G_q** FRET sensor. Cyan and yellow fluorescent protein (CFP, YFP) are fused to the α and γ subunit of the heterotrimeric G protein [169, 170] and the sensor is illuminated with light of 420 nm wavelength. In the inactive state, CFP and YFP are in close proximity and energy transfer from the CFP donor to the YFP acceptor (FRET) occurs, leading to an emission of light in a 535 nm range. Upon activation the subunits dissociate, leading to loss of energy transfer. Emission from CFP therefore increases, accompanied by a drop in YFP fluorescence. This is shown in the time traces. The ratio between YFP and CFP emission was calculated and taken as a measure of **G_q** activation. B) HEK293 cells stably expressing the CaSR were transfected with the **G_q** FRET sensor and exposed to different stimuli. The stimuli we chose fall into three groups: i. direct activators, ii. allosteric, CaSR specific drugs and iii. modulators that acted in combination with Ca^{2+} as main stimulus. The CaSR ligands considered in this study were chosen from three groups: i. direct activators (agonists), ii. allosteric, CaSR-specific drugs, and iii. (allosteric) physiological modifiers that typically act in combination with Ca^{2+} as main stimulus (Fig. 3.1B).
During the experiments, the cells were exposed to increasing doses of agonists, in the presence or absence of CaSR-drugs or modifiers, unless stated otherwise.

We monitored the responses of single cells to increasing ligand concentrations in real-time. Figure 3.2 gives an overview of the whole procedure. The $G_q$ activation in individual cells in response to a step-wise increase in the external calcium concentration was measured over time. (Later we repeated this experiment with other ligands and their combinations.) Steady-state $G_q$ activation is reached when the FRET ratio becomes constant. Steady states were found after each step increase of the calcium concentration. From the response curves of individual cells, mean traces and standard errors (SEM) were calculated. The dose-response relation was obtained from this data, it relates the steady-state values of $G_q$ activation to the varied agonist concentration, in the presence or absence of additional ligands.

The consecutive stimulation of the cells with the step-wise increase of calcium concentrations resulted in comparable $G_q$ activity as single stimulations with different calcium concentrations did (figure S3.7). This indicates that dose response curves of single cells can be obtained by step-wise sequential stimulation.

To test whether the CaSR-expressing HEK293 cells can respond to CaSR stimuli independent of CaSR, we carried out an experiment with HEK293 wild-type cells, which do not endogenously express the CaSR [171]. We confirmed that these cells indeed did not show $G_q$ responses when exposed to CaSR stimuli (panels D in figures S3.8, S3.9, S3.11 - S3.15).

We note that at low extracellular Ca$^{2+}$ concentrations, several CaSR-HEK cells responded to the calcium with a transient overshoot in $G_q$ activation. The $G_q$ activation signal then increased before it settled to a lower steady-state level. This temporal activation peak can lead to transient signaling to downstream targets, such as PLC. We can therefore not exclude that transient downstream signaling events can occur in response to external calcium, even when hardly any $G_q$ response is indicated by the dose-response curve of active steady-state $G_q$ as function of the agonist concentration.

We found an EC$_{50}$ value of 3.4 mM Ca$^{2+}$, which fits perfectly within the range of reported values ($\approx$ 3 - 4 mM), obtained with intracellular calcium mobilization assays in CaSR-HEK cells [62, 63, 172, 173]. In similar assays, Hill coefficients ($n_H$) were found to range from approximately 3 to 6 [52, 66, 172, 174]. Hill coefficients are an appropriate measure to quantify the sensitivity of a multistep protein-ligand binding event [175]. A Hill coefficient greater unity indicates that bound ligands make the receptor more susceptible for binding of further ligand molecules, the reverse for coefficients below unity. We found a Hill coefficient of 4.2 in this study which fits well to the earlier findings. The FRET ratio change at maximal stimulus concentration is 26.7% for stimulation with calcium (n=17). This information is summarized for all treatments in figure 3.6.
Figure 3.2: Workflow of the experimental data analysis, using an example of CaSR stimulation with Ca\(^{2+}\). CaSR-HEK cells transfected with the G\(_q\) FRET sensor were calcium depleted to a basal Ca\(^{2+}\) concentration of 0.5 mM prior to stimulation with increasing concentrations of external Ca\(^{2+}\) (0.5 - 7 mM). Dual wavelength fluorescence emission of single cells was recorded in real-time and FRET ratio (YFP/CFP) was calculated as response to the stimulus (left panels). Breaks in these fluorescence traces of the single cells result from pipetting in the next stimulus concentration. In the traces shown here, we eliminated these artifacts intentionally. The mean and standard error of these time traces was calculated for the individual treatments for n = 17 cells (middle). CFP emission: cyan; YFP emission: yellow; YFP/CFP ratio: red; the error bars indicate SEM for n = 17. The dose response curve was obtained from the steady states of G\(_q\) activity, adapted to the increased stimulus concentration in the time course (right). Open circles represent individual cells, filled circles the average of the YFP/CFP ratio; error bars indicate SEM. The solid line is the fit to equation 4.1.

**G\(_q\) activation by CaSR agonists**

The CaSR can be activated directly by a broad range of agonists in the absence of calcium, leading to distinct biased activations of downstream signaling readouts, such as IP\(_1\) accumulation, ERK1/2 phosphorylation and cAMP reduction [78]. We tested whether the biasing effect of different agonists is already established by CaSR by investigating the occurrence of biased activation of G\(_q\). When no bias at the level of the receptor and G protein is established, the biased activation of downstream readouts results from the signaling network beyond the GPCR.

We compare the CaSR-mediated activation of G\(_q\) when CaSR is activated by alterna-
Biased activation of $G_{aq}$ by the CaSR

tive agonists – at a basal level of calcium. (We require this basal concentration of $Ca^{2+}$ to guarantee healthy and coverslide-attached cells.) We have selected direct CaSR activators with different traits: i. strontium ($Sr^{2+}$), a divalent ion, and ii. two polyamines, spermine and spermidine.

$Sr^{2+}$ is less potent in $G_{aq}$ activation via CaSR than $Ca^{2+}$

Even though strontium and calcium are both divalent ions, it is unclear whether they bind to the same site of the CaSR [176]. We tested $Sr^{2+}$ concentrations from 0 - 11 mM at a basal calcium concentration of 0.5 mM. We found that $Sr^{2+}$ is less potent in activating $G_{aq}$ via CaSR than $Ca^{2+}$ is. The $G_{aq}$-vs-$Sr^{2+}$ dose-response curve (figure 3.3A) is right shifted compared to the $G_{aq}$-vs-$Ca^{2+}$ curve, with an $EC_{50}$ value of 5.9 mM and a Hill coefficient of 3.2. The response to 7 mM $Sr^{2+}$, the maximal concentration used for calcium treatment, was only about 12.6%, whereas at higher concentrations the maximal response approached that of calcium. Similar results have been found in a study of CaSR-HEK cells [177], a lower efficacy was found for $Sr^{2+}$, compared to $Ca^{2+}$, for mobilization of intracellular calcium and accumulation of inositoles. A study in medullary thyroid carcinoma cells of rats reported that $Sr^{2+}$ induced biased signaling of the CaSR with respect to the phosphorylation of ERK1/2 [168, 177]. Our data suggests that these biasing effects of $Sr^{2+}$ are already manifested at the CaSR-to-$G_{aq}$ level, resulting in a decreased CaSR-induced activation of $G_{aq}$.

We note that we found a strong covariance between the maximal activation parameter ($\beta$) and the $EC_{50}$ in the parameter fitting procedure, summarized in figure 3.6. As a result, we cannot independently estimate those parameters, their ratio we can however estimate with much higher certainty. The reason is that most of the cells did not yet reach maximal activity at the maximal strontium concentration of 11 mM, which explains the large uncertainty in the $EC_{50}$ values reported in figure 3.6.

Spermine is more potent than spermidine in $G_{aq}$ activation via the CaSR

The polyamines spermine and its precursor molecule, spermidine, are synthesized by eukaryotes and act as allosteric CaSR agonists [78, 174] with $EC_{50}$ values for intracellular calcium mobilization of 200 - 500 $\mu$M (spermine) and about 4 mM (spermidine) [174]. We tested a spermine concentration range of 0 - 5 mM and for spermidine we took 0 - 13 mM. Spermine and spermidine activated $G_{aq}$, albeit to different extents (figure 3.3B).

Spermidine has a low efficacy for $G_{aq}$ activation via CaSR; it gave only a maximal ratio change of about 6.1% at 7 mM (compared to 26.7% in the calcium treatment) and a high, hard-to-estimate $EC_{50}$ value. The Hill coefficient of 1.3 suggests noncooperative binding. Because of the small effects of spermidine on CaSR-mediated $G_{aq}$ activation, we could not estimate the kinetic parameters very accurately.
Figure 3.3: Comparison of CaSR agonists with respect to their potential to activate \( G_q \). CaSR-HEK cells transfected with the \( G_q \) FRET sensor were calcium depleted to a basal \( \text{Ca}^{2+} \) concentration of 0.5 mM prior to stimulation and fluorescence emission of the \( G_q \) sensor was recorded. A) FRET ratio change comparison between \( \text{Ca}^{2+} \) (blue) and \( \text{Sr}^{2+} \) (grey). Agonists were added every 90 seconds, resulting total \( \text{Ca}^{2+} \) concentrations were 0.5, 1, 2, 3, 4, 5, and 7 mM; total \( \text{Sr}^{2+} \) concentrations were 0, 1, 3, 5, 7, 9, and 11 mM. The curve of the \( \text{Sr}^{2+} \) treatment is statistically different to the \( \text{Ca}^{2+} \) titration curve with a p-value <0.05 resulting from the inverse lack-of-fit test described in the section materials and methods. Solid lines: fit to equation 4.1. B) Comparison of CaSR-HEK cells stimulated with increasing doses of \( \text{Ca}^{2+} \) (blue), spermine (orange) and spermidine (purple). Total \( \text{Ca}^{2+} \) concentrations were the same as in A; total spermine and spermidine concentrations were 0, 0.1, 0.25, 0.5, 1, 2, 3, and 5 mM and 0, 1, 3, 4, 7, 9, 11, and 13 mM, respectively. The spermidine titration curve was significantly different to the \( \text{Ca}^{2+} \) titration with a p-value <0.05 resulting from the inverse lack-of-fit test described in the section materials and methods. Solid lines: fit to equation 4.1. In all plots, error bars indicate SEM. \( n_{\text{Ca}^{2+}} = 17; n_{\text{Sr}^{2+}} = 32; n_{\text{spermine}} = 35; n_{\text{spermidine}} = 16. \) For a summary of the statistics of these fits see figure 3.6.

In contrast, spermine has a very high potency in activating the CaSR, even higher than calcium, as determined by the \( EC_{50} \) value of 1.6 mM, but efficacy is lower (ratio change at 7 mM = 21.1%).

Our data suggests that the tested polyamines have a lower effect on CaSR-mediated \( G_q \) activation than reported in other studies on responses of downstream signaling readouts. Nevertheless, we observed a higher potency of spermine than external calcium to activate \( G_q \), which is qualitatively similar to earlier studies [174]. Spermine and spermidine, as direct CaSR-activators, have been reported to have a high potency with respect to intracellular calcium release, at a basal calcium concentration of 0.5 mM in the medium [174]. Comparing these data with our findings on \( G_q \) activation
suggests that the increased intracellular calcium release is either established through downstream processes within the signaling network, or are induced independently of \( G_\eta \).

**CaSR drugs bias the \( G_\eta \) response to \( \text{Ca}^{2+} \)**

We used synthetic, allosteric modifiers, so-called CaSR-drugs, in combination with external calcium to investigate interactions of the modifiers with external calcium at the level of \( G_\eta \) activation. Cells, pretreated with 1 \( \mu \)M of the allosteric CaSR-inhibitor (calcilytic) NPS-2143, 90 seconds prior to external calcium addition, showed reduced \( G_\eta \) activation for all considered calcium doses, resulting in a decreased maximal ratio change (16.8%; figure 3.4, red and figure 3.6). We found that NPS-2143 causes a right shift of the \( G_\eta \)-vs-\( \text{Ca}^{2+} \) dose response curve (Figure 3.4, red), increasing the \( EC_{50} \) value from 3.4 to 4.7 mM. The sensitivity, quantified with \( n_E \), reduced to 3.4 (cf. fig. 3.6). NPS-2143 is documented as a CaSR-specific inhibitor that affects intracellular calcium mobilization by decreasing the potency of extracellular calcium, resulting in a right shift of the dose-response curve of the measured intracellular calcium [59, 66]. This is in agreement with our findings.

In contrast to NPS-2143, an allosteric inhibitor, NPS-R568 is a CaSR-specific allosteric activator (calcimimetic). It potentiates the effect of external calcium stimuli on intracellular calcium release, resulting in a left-shift of the dose-response curve [58, 66]. We added 1 \( \mu \)M R-568 to the cells, again 90 seconds prior to the step-wise stimulation with external calcium. We observed \( G_\eta \) activation even at basal calcium concentrations of 0.5 mM, which is clearly visible in the dose-response curve (figure 3.4, yellow). The dose-response curve is strongly left-shifted, which is reflected by a reduced \( EC_{50} \) value to about 0.1 mM. Additionally, the maximal response is slightly elevated (31.0%), compared to stimulation with calcium alone. A near-maximal response was already established at an external calcium concentration of approximately 3 mM (Fig. 3.4, yellow). Our findings agree with known effects of NPS-R568, as the potentiating effect has been reported for a stimulus range of 0.5 to 1.5 mM, albeit without influences on maximal responses [58].

**Several allosteric modifiers do not influence \( G_\eta \) activation by CaSR signaling**

Next, we tested whether known allosteric modifiers of CaSR downstream signaling readouts influence the external calcium mediated response of \( G_{\alpha \eta} \) upon CaSR activation. We tested the amino acid L-phenylalanine (PHE) and the polyamines spermine and spermidine. A fixed concentration of these modifiers was added to the basal calcium concentration of 0.5 mM prior to the stepwise, cumulative increase of the \( \text{Ca}^{2+} \) concentration up to 7 mM.
**Figure 3.4: Effect of the CaSR-drugs on $G_q$ activation.** CaSR-HEK cells transfected with the $G_q$ FRET sensor were calcium depleted to a basal $Ca^{2+}$ concentration of 0.5 mM prior to stimulation. During acquisition of fluorescence, 90 seconds before the $Ca^{2+}$ doses, 1 $\mu$M NPS-2143 (red curve) or 1 $\mu$M R-568 (yellow curve) was added to the cells and kept constant throughout the experiment. The presence of the calcimimetic R-568 resulted in a statistically significantly different curve than $Ca^{2+}$ titration alone, with a p-value $<0.05$ resulting from the inverse lack-of-fit test described in the section materials and methods. $n_{calcium} = 17$, $n_{NPS−2143} = 11$, $n_{NPS−R−568} = 26$. Solid lines: fit to equation 4.1; error bars indicate SEM. For a summary of the statistics of these fits see figure 3.6.

### L-phenylalanine as CaSR modifier does not alter $G_q$ activity

The CaSR has been reported to function as a broad range amino acid sensor, with L-phenylalanine having one of the strongest influences [165]. Millimolar concentrations of PHE turn sinusoidal oscillations in intracellular calcium into more spike-like dynamics [65]. Positive cooperativity between $Ca^{2+}$ and PHE has been reported, presumably due to the allosteric interactions of their binding sites, increasing both potency and cooperativity [165, 178]. It has been suggested $G_{12/13}$ is responsible for signaling to intracellular calcium oscillations, independent of $G_q$ [179].

We hypothesized that enhanced signaling via $G_{12/13}$, in response to PHE, should result in a right shift of the dose-response curve of $G_q$ activity in the presence of PHE, due to G protein competition. We added 10 mM PHE to the medium prior to the calcium stimulation and found that, despite the downstream signaling effects reported in literature, L-phenylalanine did not influence the CaSR in activating $G_q$. Our data does therefore agree with the earlier hypotheses that PHE signals independent of $G_q$, but we observed no evidence for G protein competition.

The $G_q$-vs-$Ca^{2+}$ dose-response curves are not significantly different in the presence or absence of the amino acid, the dose-response curve characteristics ($EC_{50} = 3.9$ mM, max. response = 24.0% and Hill coefficient = 3.7) are therefore essentially unchanged (figures 3.5A, 3.6).
Figure 3.5: Comparison of CaSR modifiers with respect to their potential to influence Ca$^{2+}$-induced $G_{q}$ activation. CaSR-HEK cells transfected with the $G_{q}$ FRET sensor were calcium depleted to a basal Ca$^{2+}$ concentration of 0.5 mM prior to stimulation. CaSR modulators PHE, spermine and spermidine were added to the assay 90 seconds before Ca$^{2+}$ stimulation, and fluorescence emission of the $G_{q}$ sensor was recorded. A) Comparison of $G_{q}$ activity upon Ca$^{2+}$ stimulation in the presence (green) and absence of 10 mM PHE (blue). B) Effects of Ca$^{2+}$ stimulation in the presence of 0.5 mM spermine (orange), 13 mM spermidine (purple) or in the absence of modifier (blue). In all plots, error bars indicate SEM. Solid lines are the fit to equation 4.1. 

$\text{n}_{\text{Ca}^{2+}} = 17, \text{n}_{\text{Ca}^{2+} + \text{PHE}} = 43, \text{n}_{\text{Ca}^{2+} + \text{spermine}} = 33, \text{n}_{\text{Ca}^{2+} + \text{spermidine}} = 25$. For a summary of the statistics of these fits see figure 3.6.

**Polyamines influence the potency and cooperativity of the Ca$^{2+}$-induced activation of $G_{q}$ by CaSR**

Quinn et al. [174] reported that the polyamines spermine and spermidine reduced the activation threshold of the CaSR for extracellular calcium, resulting in a left-shift of the dose-response curves measured in intracellular calcium assays.

We held the polyamine concentrations constant to investigate their effect as allosteric modifiers during the calcium titration experiments. We found that 0.5 mM spermine (half its EC$_{50}$ value) and 13 mM spermidine (maximal concentration used in the spermidine titration experiment) caused subtle changes in $G_{q}$ activation by Ca$^{2+}$. The presence of the modifiers slightly affected the EC$_{50}$ concentrations (2.6 mM with spermine, 2.9 mM with spermidine, 3.4 mM without modifier), and reduced the maximal ratio change to about 19.3 and 23.4 %, compared to 26.7 % without polyamines (figure 3.5B, 3.6). Interestingly, the Ca$^{2+}$ cooperativity markedly decreased, indicated by
Hill coefficients of 2.5 (spermine) and 2.7 (spermidine).

We observed that some cells responded to very low concentrations of calcium with a transiently peaked $G_q$ activation (figure S3.15, S3.16) in the presence of spermine and spermidine. Again, we emphasise that this can have a dynamic impact on downstream signaling.

Summarizing, we see subtle modifying effects of the polyamines on the Ca$^{2+}$-induced activation of $G_q$ by CaSR, leading to an altered shape of the dose-response curve. From these results, we conclude that the presence of the allosteric modifiers influences CaSR-$G_q$ interactions, presumably by shifting the balance of receptor conformations. The enhancing effect of the polyamines on intracellular calcium release [174] therefore appears to result from amplifications of pre-steady state dynamics of $G_q$ activity, not captured by the dose-response curves, or they are due to a biasing effect of the CaSR that is independent of $G_q$ activity.
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<table>
<thead>
<tr>
<th>Drugs</th>
<th>Modifiers</th>
<th>Direct CaSR-Activators</th>
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<tbody>
<tr>
<td>Ca$^{2+}$ + 1 μM NPS-2143</td>
<td>Ca$^{2+}$ + 10 mM PHE</td>
<td>Ca$^{2+}$ + 0.5 μM spermine</td>
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<tr>
<td>Ca$^{2+}$ + 10 mM spermine</td>
<td>Spermine</td>
<td>Ca$^{2+}$ + 13 mM spermidine</td>
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<tr>
<td>Sr$^{2+}$</td>
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Figure 3.6 (previous page): Overview of the signaling kinetics for the different CaSR-treatments. Hill coefficients ($n_H$), and EC$_{50}$ values of the different CaSR-treatments were obtained by fitting the dose-response data of the single cells within the different treatments to equation 4.1. Maximum ratio change (YFP/CFP) of the single cells corresponding to 7 mM agonist was calculated upon the parameter values obtained by the fitting. These characteristic values are represented with box plots. Upper plot: EC$_{50}$ values [mM]. Middle: Hill coefficients. Lower plot: YFP/CFP ratio changes (in %), corresponding to stimulation with 7 mM agonist. Boxes: interquartile range (IR) from the 25$_{th}$ and 75$_{th}$ percentile. White line: median. Error bars: indicating the most extreme data point within 1.5 $\cdot$ IR distance from the 25$_{th}$ and 75$_{th}$ percentile. Outliers represent data points with distance of more than 1.5 $\cdot$ IR (black) and 3 $\cdot$ IR (grey). Insets correspond to the calculated median values. The size of the sample is indicated by $n$.

3.3 Discussion

Whether ligand bias observed in signaling readouts downstream of GPCRs can be attributed to GPCRs only or, in addition, to the connecting signaling network is unclear for most GPCRs. To gain more understanding of the ligand-biasing potential of a GPCR, we measured the direct activation of G$_q$ by the class C GPCR CaSR. We will discuss our results, summarized in figure 3.6, in the light of reported biased activation of downstream signaling readouts by the CaSR.

The kinetic characterisation of activation of G$_q$ by Ca$^{2+}$ only (figure 3.2 and 3.6) is very similar to the kinetics reported with intracellular calcium mobilization as downstream read-out, which is believed to run via G$_q$ [174]. We also found the expected activation and inhibition effects of the calcimimetic (NPS-R568) and the calcilytic (NPS-2143) [58, 59], respectively, confirming that we indeed observe CaSR responses. Our G$_q$ activation responses to strontium are in line with earlier studies as well, indicating that this agonist is less potent than calcium [78, 168].

The high similarity between classical Ca$^{2+}$ mobilization data and our new upstream G$_q$ activation measurements indicate that the relay from G$_q$ to intracellular calcium (including PLC activation and IP$_3$-induced Ca$^{2+}$ channel opening at the ER) is highly linear. Hence, in these cases, G$_q$ is a reliable read-out for assessing downstream signaling activation.

At the level of G$_q$ activation, we did not observe low concentration effects of the polyamines (spermine and spermidine), which were observed earlier with intracellular calcium release data [174]. Also, the polyamides did not function as modifiers, increasing the receptor sensitivity to extracellular calcium [174]. One explanation for these discrepancies is that the connecting signaling network, with G$_q$ as input, plays an important role. For instance, it can in principle attenuate or amplify responses of G$_q$ to CaSR agonists and modifiers. Transient activity of G proteins can impact downstream signaling as well, which are not visible in our steady-state dose-response
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curves. We note that in the spermine and spermidine titration experiments, we did not observe transient activity at low agonist concentrations (figures S3.10, S3.11).

We cannot rule out that polyamines stabilize receptor conformations that signal independently of $G_{q}$, this would make them true biasing signals. Indeed, we find indications that spermine is acting as a biasing agonist. In our experiments, increases in spermine led to a disproportionate fluorescence change of CFP and YFP (figure S3.10). Whereas we generally observe a comparable quantitative change in CFP and YFP fluorescence, we measured with spermine a fast increase in CFP fluorescence that was not reflected by an evenly fast decrease in YFP fluorescence. This effect was not caused by autofluorescence of the compound (see figure S3.17B). The observed behavior might be caused by differential coupling of $G_{q}$ with $G_{q}$ subunits, or an altered binding to the receptor, for instance as multimers, suggesting that the conformation of the CaSR bound to the polyamine influences the CaSR-$G_{q}$ assembly. We observed a similar disproportional change in CFP and YFP fluorescence in the presence of the calcimimetic NPS-R-568 that was also not caused by autofluorescence (figure S3.13 and S3.17A).

We did not find any effects of the amino acid phenylalanine on $G_{q}$ activation. This confirms earlier indirect inferences that $G_{q}$ is not involved in signaling in response to amino acids [179]. What we did expect however, but did not see, is any evidence of $G$ protein competition effects in the $G_{q}$ signal upon PHE addition. This could be due to the fact that fluorescent $G$ protein that we use is ectopically expressed (as compared to $G_{12/13}$), or it suggests that no pre-coupling of $G$ proteins occurs in the absence of a signal and, hence, less competition.

Our results show that single-cell based pharmacology has great potential to complement existing high-throughput analyses. For instance, we were able to trace single cell responses to different ligands, monitor their (de-)activation responses and obtain kinetic information. This emphasizes the importance of single-cell, real-time measurements of signaling. Also large cell-to-cell variabilities have been found in cellular signaling [180, 181], indicating that the signaling outcome of the same signal added to the same type of cells, cultured in the same way, can still be different. This indicates that in some cases subpopulations of cells, with qualitatively different signaling dynamics, should even be distinguished.

Summarizing our observations, we find that ligand bias can be established at the onset of cellular signaling, at the level of a GPCR and a G protein interaction. This is likely occurring via the by-now-popular view in the field that distinct GPCR conformations, signaling via different $G$ proteins, are stabilized by particular ligand combinations. We therefore find indirect evidence in support of this model. Direct observation of distinct receptor conformations that signal differently would be a major next achievement in the field. We also found evidence that the signaling network downstream of a GPCR, linking a G protein to a downstream signaling readout, can modulate ligand bias.
We see great potential for experiments that illustrate in single living cells, in real-time, the decomposition of ligand bias into receptor and downstream-signaling-network contributions, for instance by using multiple fluorescent reporters in the same cell to study how their covariations depend on extracellular signal combinations.

3.4 Materials and Methods

Materials

Dulbecco’s Modified Eagle Medium (DMEM) + GlutaMax, calcium free DMEM, Hank’s Balanced Salt Solution (HBSS), penicillin-streptomycin (pen/strep), Lipofectamine 2000 transfection reagent and Hygromycin B were purchased from Life Technologies; the allosteric CaSR modulators R-568 and NPS-2143 from Tocris Biosciences. All other chemicals were from Sigma-Aldrich if not stated otherwise. HEK-293 wild-type cells were obtained from the American Tissue Culture Collection (ATCC® CRL-1573™). HEK-293 cells stably expressing the human calcium-sensing receptor (CaSR-HEK) were a friendly gift from Dr. Donald Ward, The University of Manchester. The plasmid containing the cDNA encoding human $G_\alpha q$, $G_\beta_1$ and $G_\gamma_2$ tagged to the fluorescent proteins are described in [169, 170].

Cell Culture & Transfection

HEK-293 cells and CaSR-HEK cells were grown in DMEM + GlutaMax supplied with 10% FCS and 1 % penicilin/streptomycin. In addition, CaSR-HEK cells were under Hygromycin B selection (200 $\mu$M/ml). Two days prior to the experiment, the cells were seeded on fibronectin-coated glass coverlips. Lipofectamine 2000 was used according to the manufacturer’s instructions to transfect the cells with plasmids encoding the FRET sensor $G_\alpha q$-CFP / $G_\beta_\gamma$-YFP the day before the experiment.

Wide-Field Fluorescent Microscopy

HEK-293 cells and CaSR-HEK cells grown on coverslips were mounted into an Attofluor cell chamber and maintained in microscopy medium (20 mM HEPES (pH = 7.4), 137 mM NaCl, 5.4 mM KCl, 0.5 mM CaCl$_2$, 0.8 mM MgCl$_2$ and 20 mM glucose). They were placed on a Zeiss inverted microscope (Axiovert 200M) equipped with an oil immersion 40x objective and kept at 37°C. The probes were illuminated with a Xenon arc lamp with a computer controlled monochomator (bandwidth 30 nm) and excited at 420 nm. Emission of the single cells was detected with a 470/30 bandpass filter and 535/30 bandpass filter by turning the filter wheel for CFP and YFP fluorescence, respectively. The images were binned (4x4) and recorded with a
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cooled charged coupled device camera (Coolsnap HQ, Roper Scientific, Tucson, AZ, USA). The illumination time was set to 210 ms for both CFP and YFP.

**Experimental Design**

Prior to the experiments, the cells were Ca\(^{2+}\)-depleted to reduce G protein activity induced by the CaSR to a minimum. The medium of the cells was changed twice with serum free DMEM containing 0.5 mM Ca\(^{2+}\) and subsequently incubated in the low-calcium medium for at least 20 min prior to experiments at 37 °C and 5% CO\(_2\). The cells were stimulated with stepwise increasing agonist concentrations every 90 seconds to allow the formation of a new steady state of \( G_{\text{aq}} \) activity. Direct agonists used were Ca\(^{2+}\), Sr\(^{2+}\), spermine and spermidine. Dependent on the experimental set up, CaSR specific drugs and modulators were added to the cells 90 seconds before the Ca\(^{2+}\) stimulation as follows: 1 µM allosteric inhibitor NPS-2143 or 1 µM calcimimetic R-568; 10 mM Phenylalanine, 0.5 mM spermine, or 13 mM spermidine. NPS-2143 and R-568 were dissolved in DMSO. It was made sure that only a total amount of 0.01 % DMSO was added to the cells.

**Image and data analysis**

ImageJ (http://rsbweb.nih.gov/ij/) has been used to analyze the FRET ratio imaging data: The average fluorescence intensity of individual cells in the CFP and the YFP channel intensity were obtained by drawing regions of interest (ROI) of the individual cells. Background intensity was obtained by selecting an ROI outside of cells. The background data was subtracted from average intensities of the cells and the data was normalized to the average of the first 20 frames without stimulus. Further, YFP data was corrected for crosstalk from CFP emission into the YFP channel (55%). These normalized intensity data were used to calculate the YFP/CFP ratio, which accounted as a measure of \( G_{\text{aq}} \) activity.

Mean intensities of CFP, YFP and YFP/CFP ratio data and standard error of the mean (SEM) were calculated from at least 10 cells, obtained in at least 6 independent experiments. For the dose response data, short periods of the steady states intermediating the stimulus additions have been chosen for which the YFP/CFP ratio is plotted against the corresponding agonist dose. Same holds true for the RFP fluorescence emission, whereas steady states were hardly obtained in these measurements and mean intensities were used for the dose-response curves. YFP/CFP ratios have been fitted to a distorted Hill equation (equation 4.1)

\[
y_{\text{model}} = \alpha + \frac{\beta x^h}{K^h + x^h}
\]  

(3.1)
for which \( x \) being the agonist concentration and \( y \) the normalized fluorescent intensity. \( K \) and \( h \) are the dissociation constant and Hill coefficient, respectively with \( \alpha \) as the basal value and \( \beta \) the distortion factor. Fitting of the complete data sets was conducted by minimizing the objective function, the weighted sum of squares residuals (equation 4.2), whereas the objective of single cell read-outs was the sum of squares residuals (equation 3.3):

\[
\text{obj}_{\text{complete}} = \sum_{i=1}^{n} \frac{(y_{\text{model},i} - y_{\text{data},i})^2}{\sigma_{\text{data},i}^2},
\]

(3.2)

\[
\text{obj}_{\text{individual}} = \sum_{i=1}^{n} (y_{\text{model},i} - y_{\text{data},i})^2.
\]

(3.3)

Here, \( y_{\text{model}} \) denotes the fluorescent intensity calculated by the model, described in equation 4.1; \( y_{\text{data}} \) are the data points for agonist concentration \( i \). \( \sigma_{\text{data},i} \) is representing the standard deviation of the data per concentration \( i \).

In order to determine the distribution of \( EC_{50} \) values, Hill coefficients and maximal responses given the data, the dose-dependent single cell read-outs were fitted individually to equation 4.1. Upon these parameters obtained, maximal responses of the single cells were calculated, corresponding to 7 mM agonist concentration. The obtained signaling characteristica are displayed in Box-and-Whisker plots (Fig. 3.6) in which the box represents 50% of the data that lies within the 25\(^{th}\) and 75\(^{th}\) percentile, defining the interquartile range (IR). The median is represented as horizontal line within the box and the whiskers are drawn to the most extreme data points that still lie within the distance of 1.5 \( \cdot \) IR from the drawn percentiles. Outliers are represented as black and grey dots, if they are \( \pm 1.5 \) \( \cdot \) IR and \( \pm 3 \) \( \cdot \) IR apart from the percentiles, respectively.

Statistical analysis for the comparison of different CaSR treatments has been performed by an inverse lack-of-fit test. Therefore, all data sets were compared to the calcium titration data using F-distribution. The sum of squares (SSq) of the two data sets were calculated as described above for equation 3.3, using the fit of the calcium titration as \( y_{\text{model}} \). The random variate \( X \) of the F-function was calculated by dividing the SSq of the second data set normalized by its degrees of freedom (\( df_2 \)) by the SSq normalized to its degrees of freedom (\( df_{\text{ref}} \)) of the reference data set (equation 3.4). The \( df_2 \) and \( df_{\text{ref}} \) were:

\[
df_2 = \text{#data points};
\]

\[
df_{\text{ref}} = \text{#data points} - \text{#parameters of fitting}.
\]
The cumulative distribution function (cdf) of the corresponding F-function was calculated for the $X$ values and corresponding p-values were determined.

$$X = \frac{SSq_2/df_2}{SSq_{ref}/df_{ref}},$$

$$p = 1 - cdf(X)$$

We used a cutoff-value of 0.05 for the statistical analysis, thus only for p-values less than 0.05 we considered the curves of the different treatments as statistically different to the calcium titration curve.

All corrections and analyses, as well as plotting and curve fitting have been carried out using Wolfram Mathematica version 10.0.
Figure 3.7: Comparison of Gq activation by single calcium additions and response to cumulative additions. CaSR-HEK cells transiently transfected with the Gq FRET sensor were kept in low calcium medium for 20 minutes before the experiment. A) The cells were stimulated with a single addition of one of three different calcium concentrations and CFP and YFP emission wavelengths were recorded. The final calcium concentrations in the medium were: 2.7, 5.2 and 7.2 mM. Mean traces of the time course data are shown for CFP (cyan), YFP (yellow) and YFP/CFP ratio (red). Error bars indicate SEM. B) Comparison of the Gq activity between single doses and cumulative additions (data from calcium titration experiment, main text). For the single stimulations, the mean responses were determined at the steady states of the time courses in A, and error bars indicate the SEM (orange). The Gq responses during the cumulative stimulation (blue) were determined by the fitting curves (equation 4.1) of the calcium-titration dose-response curves for the mentioned calcium concentrations. Error bars indicate SEM.
Biased activation of $G_{q}$ by the CaSR

Figure 3.8: Calcium titration experiment. CaSR-HEK cells transiently transfected with the $G_{q}$ FRET sensor were kept in low calcium medium (0.5 mM) for 20 minutes before the experiment. During the experiment, the cells were exposed to cumulative increasing calcium concentrations and CFP and YFP emission wavelengths were recorded. The cells were exposed to calcium concentrations of 0.5, 1, 2, 3, 4, 5 and 7 mM. The period between stimulus addition was 90 seconds. $n = 17$ cells. A) time traces of CFP (upper) and YFP (middle) emission and YFP/CFP ratio (lower panel) of the individual cells. B) Mean traces of the time course data for CFP (cyan), YFP (yellow) and YFP/CFP ratio (red). Error bars indicate SEM. C) Dose-response curves generated from the steady-state responses of CFP (upper), YFP (middle) and YFP/CFP ratio (lower panel) to the increasing stimulus concentrations. Empty circles: individual cells. Filled circles: mean values. Error bars indicate SEM. D) Negative control: HEK293 wild-type cells transfected with the $G_{q}$ FRET sensor were exposed to the same experimental setup as described above.
Figure 3.9: Strontium titration experiment. CaSR-HEK cells transiently transfected with the Gq FRET sensor were kept in low calcium medium (0.5 mM) for 20 minutes before the experiment. During the experiment, the cells were exposed to cumulative increasing strontium concentrations and CFP and YFP emission wavelengths were recorded. The cells were exposed to strontium concentrations of 0, 1, 3, 5, 7, 9, and 11 mM. The period between stimulus addition was 90 seconds. n = 32 cells. A) time traces of CFP (upper) and YFP (middle) emission and YFP/CFP ratio (lower panel) of the individual cells. B) Mean traces of the time course data for CFP (cyan), YFP (yellow) and YFP/CFP ratio (red). Error bars indicate SEM. C) Dose-response curves generated from the steady-state responses of CFP (upper), YFP (middle) and YFP/CFP ratio (lower panel) to the increasing stimulus concentrations. Empty circles: individual cells. Filled circles: mean values. Error bars indicate SEM. D) Negative control: HEK293 wild-type cells transfected with the Gq FRET sensor were exposed to the same experimental setup as described above. n = 12.
Figure 3.10: Spermine titration experiment. CaSR-HEK cells transiently transfected with the G$_q$ FRET sensor were kept in low calcium medium (0.5 mM) for 20 minutes before the experiment. During the experiment, the cells were exposed to cumulative increasing spermine concentrations and CFP and YFP emission wavelengths were recorded. Spermine concentrations used: 0, 0.1, 0.25, 0.5, 1, 3, and 5 mM. The period between stimulus addition was 90 seconds. n = 34 cells. A) time traces of CFP (upper) and YFP (middle) emission and YFP/CFP ratio (lower panel) of the individual cells. B) Mean traces of the time course data for CFP (cyan), YFP (yellow) and YFP/CFP ratio (red). Error bars indicate SEM. C) Dose-response curves generated from the steady-state responses of CFP (upper), YFP (middle) and YFP/CFP ratio (lower panel) to the increasing stimulus concentrations. Empty circles: individual cells. Filled circles: mean values. Error bars indicate SEM.
Figure 3.11: Spermidine titration experiment. CaSR-HEK cells transiently transfected with the $G_q$ FRET sensor were kept in low calcium medium (0.5 mM) for 20 minutes before the experiment. During the experiment, the cells were exposed to cumulative increasing spermidine concentrations and CFP and YFP emission wavelengths were recorded. Spermidine concentrations used: 0, 1, 3, 5, 7, 9, 11 and 13 mM. The period between stimulus addition was 90 seconds. $n = 16$ cells. A) time traces of CFP (upper) and YFP (middle) emission and YFP/CFP ratio (lower panel) of the individual cells. B) Mean traces of the time course data for CFP (cyan), YFP (yellow) and YFP/CFP ratio (red). Error bars indicate SEM. C) Dose-response curves generated from the steady-state responses of CFP (upper), YFP (middle) and YFP/CFP ratio (lower panel) to the increasing stimulus concentrations. Empty circles: individual cells. Filled circles: mean values. Error bars indicate SEM. D) Negative control: HEK293 wild-type cells transfected with the $G_q$ FRET sensor were exposed to the same experimental setup as described above. $n = 13$. 
Figure 3.12: Calcium titration experiment in the presence of the calcilytic NPS-2143. CaSR-HEK cells transiently transfected with the \(G_q\) FRET sensor were kept in low calcium medium (0.5 mM) for 20 minutes before the experiment. At the start of the experiment, the cells were exposed to 1 µM NPS-2143 and after 90 seconds incubation time calcium was increased in a step wise manner. The CFP and YFP emission wavelengths were recorded. Calcium concentrations used: 0.5, 1, 2, 3, 4, 5, and 7 mM. The period between stimulus addition was 90 seconds. \(n = 16\) cells. A) time traces of CFP (upper) and YFP (middle) emission and YFP/CFP ratio (lower pannel) of the individual cells. B) Mean traces of the time course data for CFP (cyan), YFP (yellow) and YFP/CFP ratio (red). Error bars indicate SEM. C) Dose-response curves generated from the steady-state responses of CFP (upper), YFP (middle) and YFP/CFP ratio (lower pannel) to the increasing stimulus concentrations. Empty circles: individual cells. Filled circles: mean values. Error bars indicate SEM. D) Negative control: HEK293 wild-type cells transfected with the \(G_q\) FRET sensor were exposed to the same experimental setup as described above. \(n = 3\).
Ca\textsuperscript{2+} titration + 1 µM NPS-R568

Figure 3.13: Calcium titration experiment in the presence of the calcimimetic NPS-R568. CaSR-HEK cells transiently transfected with the G\textsubscript{q} FRET sensor were kept in low calcium medium (0.5 mM) for 20 minutes before the experiment. At the start of the experiment, the cells were exposed to 1 µM NPS-R568 and after 90 seconds incubation time calcium was increased in a step wise manner. The CFP and YFP emission wavelengths were recorded. Calcium concentrations used: 0.5, 1, 2, 3, 4, 5, and 7 mM. The period between stimulus addition was 90 seconds. n = 25 cells. A) time traces of CFP (upper) and YFP (middle) emission and YFP/CFP ratio (lower panel) of the individual cells. B) Mean traces of the time course data for CFP (cyan), YFP (yellow) and YFP/CFP ratio (red). Error bars indicate SEM. C) Dose-response curves generated from the steady-state responses of CFP (upper), YFP (middle) and YFP/CFP ratio (lower panel) to the increasing stimulus concentrations. Empty circles: individual cells. Filled circles: mean values. Error bars indicate SEM. D) Negative control: HEK293 wild-type cells transfected with the G\textsubscript{q} FRET sensor were exposed to the same experimental setup as described above. n = 5.
Figure 3.14: Calcium titration experiment in the presence of phenylalanine. CaSR-HEK cells transiently transfected with the G_q FRET sensor were kept in low calcium medium (0.5 mM) for 20 minutes before the experiment. At the start of the experiment, the cells were exposed to 10 mM phenylalanine and after 90 seconds incubation time calcium was increased in a step wise manner. The CFP and YFP emission wavelengths were recorded. Calcium concentrations used: 0.5, 1, 2, 3, 4, 5, and 7 mM. The period between stimulus addition was 90 seconds. n = 43 cells. A) time traces of CFP (upper) and YFP (middle) emission and YFP/CFP ratio (lower panel) of the individual cells. B) Mean traces of the time course data for CFP (cyan), YFP (yellow) and YFP/CFP ratio (red). Error bars indicate SEM. C) Dose-response curves generated from the steady-state responses of CFP (upper), YFP (middle) and YFP/CFP ratio (lower panel) to the increasing stimulus concentrations. Empty circles: individual cells. Filled circles: mean values. Error bars indicate SEM. D) Negative control: HEK293 wild-type cells transfected with the G_q FRET sensor were exposed to the same experimental setup as described above. n = 7.
**Figure 3.15: Calcium titration experiment in the presence of 0.5 mM spermine.** CaSR-HEK cells transiently transfected with the Gq FRET sensor were kept in low calcium medium (0.5 mM) for 20 minutes before the experiment. At the start of the experiment, the cells were exposed to 0.5 mM spermine and after 90 seconds incubation time calcium was increased in a step wise manner. The CFP and YFP emission wavelengths were recorded. Calcium concentrations used: 0.5, 1, 2, 3, 4, 5, and 7 mM. The period between stimulus addition was 90 seconds. n = 35 cells. A) time traces of CFP (upper) and YFP (middle) emission and YFP/CFP ratio (lower pannel) of the individual cells. B) Mean traces of the time course data for CFP (cyan), YFP (yellow) and YFP/CFP ratio (red). Error bars indicate SEM. C) Dose-response curves generated from the steady-state responses of CFP (upper), YFP (middle) and YFP/CFP ratio (lower pannel) to the increasing stimulus concentrations. Empty circles: individual cells. Filled circles: mean values. Error bars indicate SEM D) Negative control: HEK293 wild-type cells transfected with the Gq FRET sensor were calcium depleted to a basal level of 0.5 mM and after 20 min the emission wavelengths of CFP and YFP were recorded over time. The cells were exposed to 5 mM spermine and after 90 seconds the cumulative addition of calcium was started. The traces show mean data of CFP (cyan), YFP (yellow) and the YFP/CFP ratio (red) of n = 12 cells.
Figure 3.16: Calcium titration experiment in the presence of 13 mM spermidine. CaSR-HEK cells transiently transfected with the $G_q$ FRET sensor were kept in low calcium medium (0.5 mM) for 20 minutes before the experiment. At the start of the experiment, the cells were exposed to 13 mM spermine and after 90 seconds incubation time calcium was increased in a step wise manner. The CFP and YFP emission wavelengths were recorded. Calcium concentrations used: 0.5, 1, 2, 3, 4, 5, and 7 mM. The period between stimulus addition was 90 seconds. n = 35 cells. A) time traces of CFP (upper) and YFP (middle) emission and YFP/CFP ratio (lower panel) of the individual cells. B) Mean traces of the time course data for CFP (cyan), YFP (yellow) and YFP/CFP ratio (red). Error bars indicate SEM. C) Dose-response curves generated from the steady-state responses of CFP (upper), YFP (middle) and YFP/CFP ratio (lower panel) to the increasing stimulus concentrations. Empty circles: individual cells. Filled circles: mean values. Error bars indicate SEM.
Figure 3.17: Background intensity of spermine titration experiment and calcium titration in the presence of the calcimimetic. A) CaSR-HEK cells transiently transfected with the G$_{q}$ FRET sensor were kept in low calcium medium (0.5 mM) for 20 minutes before the experiment. At the start of the experiment, the cells were exposed to 1 µM NPS-R568 and after 90 seconds incubation time calcium was increased in a step wise manner. The CFP and YFP emission wavelengths were recorded. Calcium concentrations used: 0.5, 1, 2, 3, 4, 5, and 7 mM. The period between stimulus addition was 90 seconds. The time traces of the background fluorescence of the YFP/CFP ratios do not show a change in background fluorescence after the addition of the calcimimetic. Black line: mean. Dotted lines: individual measurements. N= 18 measurements. Error bars indicate SEM. B) The CaSR-HEK cells were treated following the scheme described above, besides stimulation resulted by increasing concentrations of spermine in the absence of any CaSR modifier or drug. Spermine concentrations used: 0, 0.1, 0.25, 0.5, 1, 2, 3, 5 mM. The time traces of the background fluorescence of the YFP/CFP ratios do not show a change in background fluorescence while increasing the polyamine concentration. Black line: mean. Dotted lines: individual cells. Error bars indicate SEM.
Assessing the covariation of $G_{\alpha q}$ activity and intracellular calcium mobilization upon GPCR stimulation

In collaboration with:
Jakobus van Unen, Joachim Goedhart, Theodorus W.J. Gadella, and Frank J. Bruggeman

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Abstract

Background G protein-coupled receptor (GPCR) signaling is initiated by binding of the receptor to extracellular ligands. Different ligand combinations stabilize distinct receptor conformations that each activate intracellular signaling pathways to different degrees. This phenomenon is called biased signaling. Experimental read-outs of GPCR signaling are usually chosen downstream in the signaling network. Biased signaling that is observed at this level may therefore not solely originate from receptor-level bias but in addition from network bias that occurs downstream of the receptor.

Aim In this study, we measure the dynamic co-activation of two different signaling molecules by the external calcium sensing receptor (CaSR). We measure the direct activation of the G protein $G_q$ by CaSR and the downstream mobilization of intracellular calcium, $(Ca^{2+})_i$, upon exposure to various (allosteric) CaSR ligands. By measuring their co-activation in real time, we aim to distinguish the contributions of the receptor and the downstream network to biased signaling.

Results Using Förster Resonance Energy Transfer (FRET) and multicolor fluorescence microscopy, we measured simultaneously the dynamics of $G_q$ (de-)activation and $(Ca^{2+})_i$ mobilization in single cells, in response to distinct CaSR ligands. The co-variation of these signaling outputs allowed us to discriminate between of receptor and network contribution to biased signaling.

Conclusions We successfully co-imaged the GPCR-induced dynamics of $G_q$ and $(Ca^{2+})_i$, in single cells. The treatment with different ligands resulted in similar trends of the $G_q$-$(Ca^{2+})_i$ covariation, confirming the major role of $G_q$ in $(Ca^{2+})_i$ mobilization. We found that the precise covariation depended on the ligand combinations used. This indicates that biased signaling results at level of the CaSR and in the downstream signaling network.

4.1 Introduction

Intracellular calcium is a versatile messenger molecule that is involved in many cellular processes. Its concentration in the cytosol is regulated by the interplay of many molecular processes, involving second messengers, calcium pumps and channels [182]. Many of these processes are indirectly regulated by GPCRs.

GPCRs bind and activate distinct intracellular signaling proteins, such as heterotrimeric G proteins and $\beta$-arrestins. Different receptor conformations vary in their binding preferences for intracellular proteins ([36], reviewed in [43]). Heterotrimeric G protein binding to an active receptor induces conformational changes that lead to a GDP/GTP exchange on the $\alpha$ subunit. The $\alpha^{GTP}$ and $G\beta\gamma$ subunits then dissociate and activate downstream signaling proteins, leading to, amongst other processes, activation of intracellular calcium.
Simultaneous detection of $G_{\alpha q}$ and (Ca$^{2+}$)$_i$ covariation

Increases in intracellular calcium are induced by GPCR signaling mainly via activation of the heterotrimeric G protein $G_{\alpha q}$. The GTP-bound, active form of $G_{\alpha q}$ activates phospholipase C$\beta$ (PLC$\beta$). PLC$\beta$ cleaves phosphatidylinositol 4,5-bisphosphate (PIP$_2$) into inositol 1,4,5-trisphosphate (IP$_3$) and diacyl glycerol (DAG). IP$_3$ induces the opening of channels of the endoplasmatic reticulum (ER), the intracellular calcium store, resulting in a calcium flux into the cytosol. In addition, $G_{\beta \gamma}$ subunits of active $G_i$ proteins can also activate PLC [183, 184]. Calcium channels and pumps in the plasma membrane allow for the influx of external calcium. Crosstalk between GPCRs and plasma membrane channels has been reported in various cases, either through direct interactions or second messenger molecules [183, 185, 186] (figure 4.1).

Intracellular calcium mobilization is often used as a GPCR signaling read-out (e.g.[91, 187–189]). Different optical methods allow for the measurement of calcium in single, living cells; examples are fluorescent dyes such as Fura-2 or Fluo 4 [190], genetically-encoded fluorescent sensors from the cameleon or CaMP family [191] and bioluminescence-based assays [190]. These tools provide a rich palette of techniques that differ in measurement characteristics such as, for example, dynamic range, binding kinetics, photostability, excitation and emission wavelengths or calcium buffering capacity [190].

Since changes in free cytosolic calcium are brought about by many intracellular processes (reviewed in [182]), intracellular calcium mobilization is characterized by several complex, spatio-temporal dynamics, ranging from single channel events to (inter-)cellular calcium waves. It appears that stimulus information is encoded in the amplitude and frequency of calcium oscillations [182, 192].

GPCRs couple to many signaling pathways. Ligands activate different signaling pathways to varying degrees, a phenomenon described as ligand bias or biased agonism [70, 74–77]. The biasing ligands stabilize distinct receptor conformations that activate downstream signaling molecules to varying degrees [44, 69, 75, 193, 194]. Clinically relevant compounds have been shown to have biased activities [84, 85] and particular compounds with biasing effects have been developed [86, 87]. Most experimental studies deduce ligand bias from shifts in dose-response curves of downstream signaling read-outs [80, 94]. Since only few studies focus on the onset of ligand bias at the receptor level, it is often unknown whether ligand bias is induced by the receptor or whether it is a signaling network effect. Here we investigate the role of the network in establishing network bias.

In this study, we use the human extracellular calcium sensing receptor (CaSR). It is known for its biased signaling upon stimulation with various ligands. It is activated and allosterically modified by many external signals besides calcium, such as di- and trivalent ions, amino acids or polyamides, as well as CaSR-specific pharmaceutical compounds with activating or inhibiting effects [50, 54]. Further, it has recently been identified as an amino-acid sensor [164, 165]. Mutations in this receptor are associated with hereditary diseases related to the body calcium regulation [67, 166], as well as biased agonism resulting from gain- or loss-of-function mutations [167].
Figure 4.1: Schematic overview of the intertwined GPCR signaling pathways leading to intracellular calcium mobilization and the CaSR ligands used in this study. A) G protein-coupled receptors initiate signaling routes that lead to an increase in intracellular calcium. Activated $G_{aq}$ and $\beta\gamma$ subunits of $G_i$ proteins activate PLC, which cleaves PIP$_2$ into IP$_3$ and DAG. IP$_3$ binding to receptors in the membrane of the ER initiates calcium release from the internal stores. Further, GPCRs interact with calcium channels at the plasma membrane, allowing calcium influx to the cytosol from the extracellular space. B) HEK293 cells stably expressing the CaSR were transfected with the $G_q$ FRET sensor and the red fluorescent calcium sensor R-GECO-1 and exposed to different stimuli. The stimuli we chose fall into three groups: i. direct activators, ii. allosteric, CaSR specific drugs and iii. modulators that act in combination with external Ca$^{2+}$ as main stimulus. Dynamics of $G_q$ and (Ca$^{2+}$), were recorded.

The three families of heterotrimeric G proteins $G_{aq}$, $G_{ai}$ and $G_{a12/13}$ are activated by the CaSR, as well as $\beta$-arrestins, and initiate signaling of several downstream targets [55]. These have been observed, for instance IP$_3$ accumulation, cAMP levels, ERK phosphorylation and changes in intracellular calcium, in order to address the biasing influence of several CaSR ligands [66, 78, 167, 168]. There has been great interest in the
Simultaneous detection of $G_{aq}$ and $(Ca^{2+})_{i}$ covariation biased signaling capacity of GPCRs, implying a great potential for the development of ‘biasing drugs’ [81–83]. However, remarkably few studies are focusing on the receptor as origin of biased signaling as mainly downstream read-outs are observed. As a consequence, it is largely unknown to what extent the receptor contributes to ligand bias and how important the underlying signaling network is in establishing and modulating signaling bias.

In this work, we addressed the propagation of biased signaling induced by the CaSR. We measured the responses of CaSR-induced $G_{aq}$ and intracellular calcium mobilization simultaneously in real-time and in single cells, upon receptor stimulation with various CaSR ligands. We used a $G_{q}$ FRET sensor, described in [169, 170], and a red fluorescent, genetically encoded calcium sensor, R-GECO-1, to elucidate the covariation of these signaling components in live cells and to assess the importance of network bias.

4.2 Results

Characterization of R-GECO-1, an intracellular calcium sensor

R-GECO-1 is a genetically encoded, red fluorescent calcium sensor belonging to the CaMP family [195]. It is dim in the absence of calcium and bright in the calcium bound state. It has a dissociation constant for calcium of 480 nM [195]. It consists of the circular permutated fluorescent protein mApple that is fused to the calmodulin binding site of chicken myosin light chain kinase (M13) and a vertebrate CaM [195], illustrated in figure 4.2A. Following calcium binding, the two CaM domains interact and induce a reorganization of the fluorescent protein, which results in an increase in fluorescence [196]. Absorption and emission wavelengths of 560 nm and 600 nm are reported, respectively, which makes them applicable for the combined use with cyan/yellow fluorescence FRET sensors, as there is hardly any overlap with their respective emission spectra (figure 4.2B).

In order to determine fluorescent crosstalk between the emission channels, CaSR-HEK cells were transfected with R-GECO-1 and exposed to wavelengths for CFP (420 nm) and RFP (570 nm) excitation. Fluorescence emission was recorded in the CFP, YFP and RFP channel (470/30, 535/30 and 620/60 bandpass filters, respectively). The crosstalk of R-GECO-1 was assessed as 2.6 % and 2.4 % for the CFP and YFP emission, respectively (see Fig. 4.3A). However, the same set-up shows a decrease of RFP fluorescence (photobleaching) over the time course of a typical experiment (about 800 seconds, see Fig. 4.3B, upper pannel). In order to correct for this effect, the data was fitted to an exponential decay function (see equation 4.3) and the decay parameters determined were used to correct further R-GECO-1 data for bleaching. Interestingly, R-GECO-1 transfection also affects CFP and YFP emissions over time. The average CFP emission increases slightly over time, whereas YFP emission decreases in the
first 100 seconds and stays constant thereafter (Fig. 4.3B, middle and lower panel, respectively).

![Diagram of fluorescent sensors](image)

**Figure 4.2: Overview of the fluorescent sensors R-GECO-1 and G\textsubscript{q} FRET.** A) Schematic of the amino-acid sequence of R-GECO-1 (left), according to [195], and the G\textsubscript{q} FRET sensor (right) as described in [169, 170]. B) Emission spectra of R-GECO-1 (left) in high-calcium conditions (stimulated cells, solid line) and low-calcium conditions (non-stimulated cells, dashed line). Right: relevant excitation and emission spectra of the fluorophores in the G\textsubscript{q} FRET sensor. Cyan: absorption and emission spectra (lighter and darker line, respectively) of mTurquoise; Orange: emission spectrum of YFP (FRET).

**R-GECO-1 compared to the established calcium indicator Ycam**

In order to test if R-GECO-1 captures the calcium dynamics as good as other established, genetically encoded calcium sensors of the cameleon family, we transfected CaSR-HEK cells either with R-GECO-1 or the cyan/yellow fluorescence based FRET sensor Ycam. The CaSR was stimulated by sequentially increasing external calcium concentrations and the emission of both sensors was recorded. For both sensors, we found similar calcium dynamics that can roughly be clustered into for types of cellular responses: i) no response, ii) steady increase, iii) spiking, and iv) oscillations. In figure 4.4 representative responses of intracellular calcium dynamics in single cells are displayed for R-GECO-1 transfection (Fig. 4.4A) and transfection with Ycam (Fig. 4.4B). The cells either hardly showed any reaction to stimulation (left panel), rapid increases upon higher stimulus concentration and more or less stable fluorescence between two stimuli (second panel from left), single calcium spikes at low stimuli and damped oscillations at higher concentrations (second panel from right), and sustained oscillations for external calcium concentrations between 3 and 5 mM (right panel). In the R-GECO-1 transfected cells, basal fluorescence displayed a tendency to increase, whereas such a behavior was not observed in Ycam transfected cells. Further, the distinct responses were observed to different percentages for the two constructs (Fig. 4.4C). A higher percentage of cells transfected with Ycam displayed
Simultaneous detection of $G_{\alpha q}$ and $(Ca^{2+})_i$ covariation

**Figure 4.3: Photophysical effects of R-GECO-1.** A) Fluorescent crosstalk (bleed-through) analysis of R-GECO-1. CaSR-HEK293 cells were transiently transfected with the R-GECO-1 construct. The cells were exposed to wavelengths for CFP (420 nm) and RFP (570 nm) excitation and fluorescence emission was recorded in the CFP, YFP and RFP channels (470/30, 535/30 and 620/60 bandpass filters, respectively). R-GECO-1 shows 2.6% and 2.4% crosstalk with the CFP and YFP channel, respectively. Representative images of two cells are shown, crosstalk was calculated as the mean from 8 cells. B) Bleach curve of R-GECO-1. CaSR-HEK293 cells transfected with R-GECO-1 were exposed to CFP and RFP excitation wavelengths and emission in the RFP, CFP and YFP channel was recorded over 800 sec. Colored dots: individual cells. Black dots: Mean. Red, solid line in the RFP ex/em plot: fit to exponential decay, see equation 4.3.

sustained or damped oscillations and fewer non-responding cells compared with R-GECO-1 transfections. Despite these discrepancies, both sensors recorded qualitatively comparable dynamics and also seem to exhibit similar sensitivities for intracellular calcium as characteristic $(Ca^{2+})_i$ events, as initial rises, peaks or sustained oscillations are recorded at same stimulus levels. From this we concluded that R-GECO-1 is reliably recording intracellular calcium dynamics.
Figure 4.4: Comparison of R-GECO-1 and Ycam as intracellular calcium sensors. CaSR-HEK293 cells were transiently transfected with the R-GECO-1 or the Ycam construct. The cells were calcium depleted for at least 15 min prior to the experiment and subsequently stimulated with step-wise increasing external calcium concentrations up to 7 mM and 10 µM ionomycin as positive control at the end of the experiments. Fluorescence emission was recorded throughout the experiment. A) normalised R-GECO-1 fluorescence, B) the normalised YFP/CFP fluorescence ratio of the Ycam sensor. Cellular responses upon the stimulation were comparable for both sensors and could be clustered in four typical responses, from left to right: no response, increase, spiking and oscillations. C) The distribution of responses in percentage for a total of 161 R-GECO-1 (left) and 75 Ycam (right) transfected cells, respectively.

Simultaneous measurements of $G_q$ and intracellular calcium

We co-transfected the CaSR-HEK cells with R-GECO-1 and the $G_q$-FRET sensor to test whether we could trace changes in $G_q$ activity and intracellular calcium simultaneously. The cells were calcium depleted to a basal concentration of 0.5 mM and stimulated with a pulse of high external calcium. After a period of lower calcium concentration in the medium, a second stimulus with varying CaSR ligands was added. Ionomycin-induced internal-calcium release was used as a positive control (Fig. 4.5). As can be seen in the time-course plots and displayed images of the cells, CaSR activation by an external calcium pulse leads to an immediate, sustained activation of $G_q$, followed by a fast deactivation back to base line levels, as soon as the external calcium signal was reduced (upper images in figure 4.5A, left plots in figure 4.5B-E). The second stimulation again results in a very fast, sustained activation, its magnitude dependent on the stimulus strength (e.g. compare figures 4.5B and C). Similarly, intracellular calcium increases about 4-6 fold from its basal level upon the first stimulus...
pulse, and returns quickly to basal levels as soon as the stimulus level drops (lower images in figure 4.5A and right plots in figure 4.5B-E). However, in contrast to \(G_{aq}\) activity, the cells respond to the polyamine stimulation with a transient peak in \((\text{Ca}^{2+})_i\), that is shorter in duration and amplitude than the peak upon the first external calcium stimulation. Possibly, the \((\text{Ca}^{2+})_i\) response to calcium stimulation is a combination of calcium influx and CaSR-induced signaling, whereas the \((\text{Ca}^{2+})_i\) increase to polyamine stimulation is a signaling specific response.

Interestingly, even when the external calcium concentration is increased during a second stimulation, \((\text{Ca}^{2+})_i\) does not reach the same amplitude height observed initially. It shows a more transient behavior (see figures 4.5D and E). We did not observe substantial differences in the intracellular calcium response in the presence of calcium and phenylalanine (figure 4.5E), compared to calcium alone (figure 4.5D).

These results demonstrate that we are able to trace the comprehensive dynamics of both \(G_{aq}\) and intracellular calcium simultaneously in single cells.
Simultaneous detection of $G_{q}$ and ($Ca^{2+}$)$_{i}$ covariation

Figure 4.5 (previous page): Simultaneous acquisition of $G_{q}$ activities and intracellular calcium mobilization upon CaSR activation. CaSR-HEK cells were transfected with the $G_{q}$ FRET sensor and R-GECO-1 and calcium depleted to 0.5 mM basal calcium concentration in the medium before the experiments. The cells were stimulated with a pulse of 5 mM external calcium, followed by a second treatment with different CaSR-specific ligand or calcium and ionomycin at the end of the experiments. Fluorescence emission of both sensors in the single cells was recorded over time. A) $G_{q}$ ratio fluorescence (left image: widefield image, others: YFP/CFP ratio, green: low activity, red: high activity) and R-GECO-1 fluorescence (widefield) of representative cells at time points of characteristic dynamics. Experimental set-up as depicted in B. B) time courses of $G_{q}$ activity (left) and intracellular calcium (R-GECO-1, right) with 13 mM spermidine as second stimulus. Cells from this experiment are depicted in A. C, D, E) same set-up as in B, with 0.5 mM spermine, 5 mM Ca$^{2+}$, and 5 mM Ca$^{2+}$ with 10 mM PHE as second stimulus, respectively. Cyan, yellow and red solid lines are depicting mean fluorescence of CFP, YFP and YFP/CFP ratio of the $G_{q}$ FRET sensor; colored lines in the right plots display individual cells and the black, solid line depicts the mean value. Error bars represent SEM. 6 to 18 individual cells were observed for the different conditions.

Titration experiments

Next, we assessed biased signaling by monitoring the covariation of $G_{q}$ and intracellular Ca$^{2+}$, two signaling read-outs at different layers within the signal-transduction cascade that responds to CaSR ligands. To achieve this we exposed the doubly transfected CaSR-HEK cells to increasing dosages of agonist, in the presence or absence of allosteric CaSR modifiers.

In this study, we made use of calcium and strontium as direct agonists. The amino acid L-phenylalanine (PHE), as well as the CaSR-specific, synthetic allosteric ligands NPS-2143 (calcilytic) and NPS-R-568 (calcimimetic) were used in combination with external calcium as main stimulus.

During the titration experiments, we monitored the responses of the single cells to the rising ligand concentrations in real time. These were used to calculate mean traces and standard errors (SEM). We observed steady-state $G_{q}$ activation after the single agonist additions as periods of constant fluorescence. These steady-state levels were related to the varied agonist concentrations in order to determine the dose-response relationships of the different treatments.

In most cases, we did not observe steady-states of the intracellular calcium. Therefore, we used the mean fluorescence of a defined period in between agonist additions to establish the dose-response curves. A graphical summary of the procedure is displayed in figure 3.2 in the previous chapter.

In figure 4.6 the time-courses and dose-response curves of $G_{q}$ activity and intracellular calcium upon external calcium stimulation are depicted as an example; similar curves were obtained for the experiments with the different ligands and modifier combinations (see supplementary information).

Due to the influence of R-GECO-1 transfection on the CFP and YFP emission channels reported earlier, we note that the results of the simultaneous measurements ($G_{q}$ and
Figure 4.6: Time course and dose-response curves of Gq and intracellular calcium upon calcium-titration. CaSR-HEK cells were transfected with the Gq FRET sensor and R-GECO-1 and calcium depleted to 0.5 mM basal calcium concentration at least 15 min prior to the experiments. The cells were stimulated with increasing agonist concentrations, in this case external calcium up to 7 mM. Ionomycin served as positive control at the end of the experiments. Fluorescence emission of both sensors in the single cells was recorded over time. Time courses (left panels) and dose-response curve (right panels) of Gq (upper panels) are depicted as means of the individual cells, the colored, open circles display the responses of the individual cells. Responses of individual cells (colored lines and open circles, respectively) as well as mean traces (black, solid line and black, filled circles) are shown for the intracellular calcium (lower panels). Error bars indicate SEM. Red, solid line in the Gq dose-response curve: fit to equation 4.1.

(Ca^{2+})_i are not directly comparable with the results obtained when only the Gq-FRET sensor was used (chapter 3). The two systems, however, give qualitatively similar results, see figure 4.7.

Covariance of intracellular calcium mobilisation and Gq activation

Next, we compared results obtained from the double transfection experiments, by comparing cells with the same transfection properties, to investigate network bias that occurs downstream of Gq and upstream of intracellular calcium mobilisation. In figure 4.8, we compare the mean values of both read-outs for different CaSR ligands and stimulus combinations. We plotted the normalized R-GECO-1 intensity, the indicator for (Ca^{2+})_i, as function of the Gq activity. Since Gq is upstream of (Ca^{2+})_i, Gq can be viewed as an input and (Ca^{2+})_i as an output. Inputs of Gq are then all
Simultaneous detection of G\textsubscript{q} and (Ca\textsuperscript{2+})\textsubscript{i} covariance

Figure 4.7: Comparison of the effects of single versus double transfection on time courses and dose-response curves. CaSR-HEK cells were either transfected with the G\textsubscript{q} FRET sensor alone (lighter colors), or with the G\textsubscript{q} FRET sensor and R-GECO-1 simultaneously (darker colors). After calcium depletion to 0.5 mM basal calcium concentration, the cells were stimulated with increasing agonist concentrations, in the absence of CaSR-modifiers (upper panels) or in the presence of 1 µM of the calcimimetic NPS-R-568 (lower panels). Fluorescence emission of the single cells was recorded over time. Time courses (left panels) and dose-response curves (right panels) of G\textsubscript{q} are depicted as means of the individual cells. Lighter blue: calcium titration in solely G\textsubscript{q} transfected cells; darker blue: calcium titration in doubly transfected cells. Lighter orange: calcium titration in the presence of 1 µM calcimimetic of solely G\textsubscript{q} transfected cells; darker orange: calcium titration in the presence of 1 µM calcimimetic of doubly transfected cells. Error bars indicate SEM.

ligands used to activate CaSR.

A calcium titration (blue, all plots in figure 4.8) induces an intracellular calcium increase, even when no G protein activity was recorded, followed by a slight increase of G protein activity. At slightly higher concentrations of external calcium, the (Ca\textsuperscript{2+})\textsubscript{i} response increases with G\textsubscript{q} activity, after which it settles to a slightly lower level.

In the presence of the calcilytic (NPS-2143) and the calcimimetic (NPS-R-568) (fig. 4.8A, red and yellow, respectively), the intracellular calcium release is reduced in comparison to the Ca\textsuperscript{2+} titration alone. The calcilytic also blocks G\textsubscript{q} activity while the calcimimetic enhances it. Further, intracellular calcium mobilization is induced by the calcimimetic even at basal calcium concentrations in the medium (see also supplementary information, figure 4.13).

Strontium is also less potent in activating G\textsubscript{q} and releasing intracellular calcium. It reaches however similar G\textsubscript{q} activation levels as external calcium at high concentrations (figure 4.8B, black).

In the presence of 10 mM phenylalanine (fig 4.8C, green), the (Ca\textsuperscript{2+})\textsubscript{i}-vs-G\textsubscript{q} relation is
Figure 4.8: Direct comparison of Gq activity and intracellular calcium release as response to different stimuli. CaSR-HEK cells transiently transfected with both Gq-FRET sensor and R-GECO-1 were stimulated with increasing concentrations of CaSR-specific ligands, as described in section earlier. Gq activity and internal Ca\(^{2+}\) release were measured simultaneously and the data was processed as described. Dose-response data of Gq activity and intracellular calcium for the increasing stimulus doses is plotted against each other. A) comparison of the CaSR-drugs. Blue: Ca\(^{2+}\) titration, red: Ca\(^{2+}\) titration + 1 µM CaSR inhibitor NPS-2143, yellow: Ca\(^{2+}\) + 1 µM CaSR activator NPS-R568. B) CaSR activators Ca\(^{2+}\) (blue) and Sr\(^{2+}\) titration (black). C) Comparison of Ca\(^{2+}\) titration (blue) with Ca\(^{2+}\) titration in the presence of 10 mM L-PHE (green). Error bars indicate SEM. Mean results from at least 25 cells are shown.

right-shifted. (Ca\(^{2+}\))\(_i\) is less sensitive to Gq at low concentrations of calcium.

All (Ca\(^{2+}\))\(_i\)-vs-Gq curves follow a comparable trend: a steep increase in (Ca\(^{2+}\))\(_i\) mobilization at low Gq activity levels, when extracellular calcium or strontium are at low concentrations, followed by an increasing G protein activity, at intermediate extracellular calcium concentrations, and a slight reduction in (Ca\(^{2+}\))\(_i\) at high Gq.
activity when extracellular calcium is high.

We conclude that \( G_q \) is an upstream regulator of calcium mobilization, in response to CaSR activation. Since the precise (\( \text{Ca}^{2+} \))\text{v}s-\( G_q \) relation depends on the combination of CaSR-ligands, we conclude that CaSR-level as well as downstream signaling network bias occurs.

### 4.3 Discussion

GPCRs display ligand-biased signaling when exposed to various ortho- and allosteric ligands and combinations thereof. It is however largely unknown how the interplay between receptor and downstream players of signal transduction is affecting biased signaling and how the bias introduced at the level of the receptor is propagated through the downstream signal transduction network. To address this question, we aimed to visualize the activity of two different read-outs of signal transduction upon CaSR activation and explore their covariation upon receptor activation by different ligands. We used two fluorescent sensors that enabled the time-resolved monitoring of \( G_q \) activity and intracellular calcium mobilization, which are two signaling read-outs that are localized at different levels of CaSR signaling. \( G_q \) activity is a more direct receptor read-out, whereas intracellular calcium is a downstream read-out, a node in several interacting and intertwining signaling pathways.

In this work, we showed for the first time the dynamics of two GPCR signaling read-outs, simultaneously in single cells. We were able to capture the covariation of two read-outs in response to different ligands. In these studies (Fig. 4.8), intracellular calcium responded in several treatments even though we did not detect a change in \( G_q \) activity. This could have to do with a higher sensitivity of R-GECO-1 relative to the \( G_q \) FRET sensor, or with the amplification of the signal occurring before the internal calcium release. In agreement with the experiments where we transfected the cells only with the \( G_q \) sensor (chapter 3), and with previous studies [177], strontium is less potent in triggering \( G_q \) activation and also of (\( \text{Ca}^{2+} \))\text{r} release. This reduced potency in mobilizing intracellular calcium might be due a reduced influx of calcium, from the medium into the cell, upon stimulation with strontium, as during those experiments extracellular calcium is kept low at 0.5 mM. We found that the calcilytic (NPS-2143) inhibits both CaSR-induced \( G_q \) activation as well as (\( \text{Ca}^{2+} \))\text{r} mobilization, which is consistent with previous findings [59]. The calcimimetic (NPS-R-568) already triggered intracellular calcium release and \( G_q \) activation at basal calcium concentrations in the medium, and did not show further enhancing effects at high stimulus concentrations (Fig. 4.8A), which is in good agreement with [58]. We clearly see an effect of PHE on the mobilization of intracellular calcium (Fig. 4.8C). The intracellular calcium release response is right shifted to higher \( G_q \) activity levels and higher calcium stimulus concentrations, respectively. The time courses of intracellular calcium in absence and presence of phenylalanine (cf. figures S4.9 and S4.11) resemble earlier measure-
ments [178]. These results are pointing towards the hypothesis that the amino acid triggers different signaling routes, independent from $G_q$, as described in [65, 179].

This is a clear indication that downstream processes are involved in the release of intracellular calcium, on top of CaSR activity, which implies network-level bias.

A similar trend can be seen in all covariation plots: a steep intracellular calcium mobilization at low levels of $G_q$ activity, followed by constant or slightly diminished $(Ca^{2+})_i$ concentrations at higher $G_q$ activity. This confirms $G_q$ as a major regulator of intracellular calcium mobilization. Nevertheless, $G_q$ is ectopically expressed in these studies and it therefore potentially triggers signaling towards calcium mobilization. However, the $(Ca^{2+})_i$-$G_q$ relation does depend on CaSR-ligand combinations, which indicates that the signaling network downstream of CaSR determines ligand bias, in addition to CaSR itself.

We observed photobleaching of R-GECO-1 emission, in addition to influences on CFP and YFP emission over time (Fig. 4.3). Issues with the photophysical properties of R-GECO-1 have been reported earlier. In a study of Akerboom and colleagues, the sensor showed fast multi-state photobleaching and rapid photoswitching as known for its core FP mApple [197, 198]. Further, R-GECO-1 was activated also by wavelengths of 405 nm and 488 nm in addition to 561 nm, a feature that affected its emission when excitation pulses of 561 nm wavelength were applied shortly (ms-s) after pulses of 488 nm [197]. The influence of blue light on R-GECO-1 emission reported in [197] was only relevant when the second, red light excitation was applied a few milliseconds after the blue light pulse and lost significance for time intervals greater than 2.5 seconds. Such fast changes in excitation were not possible in our set-up due to the automatic changes of the filters. The fastest switch that we could maintain had a gap of five seconds between the 420 nm and 570 nm excitation. Therefore, CFP excitation should not affect R-GECO-1 fluorescence emission in our set-up. Despite the known characteristics of R-GECO-1, we observed negligible crosstalk between the channels (2.4 and 2.6 % in CFP and YFP emission channel, respectively, Fig. 4.3) in our set-up. Further, high RFP emissions in response to ionomycin treatment influenced the CFP and YFP emissions hardly (cf. figures B and D in figures S4.9 - S4.13). We conclude therefore that the R-GECO-1 signal in the CFP/YFP channel does not affect the $G_{aq}$ sensor measurements. The quantitative differences of the dose-response curves observed under single versus double transfection conditions (figure S4.14) are most probably resulting from different transfection efficiencies in the two conditions.

Possibly, the observed fluorescence emission of R-GECO-1 into the CFP channel over time (Fig/ 4.3B) is caused by green components of the fluorophore that emerge due to photoconversion or represent a fraction of proteins that remain in their immature, green state. R-GECO-1 is a non-ratiometric fluorophore, implying that the absolute fluorescence of one emission wavelength is recorded. In contrast, emissions of two distinct wavelengths are recorded and a ratio of these two is obtained as read-out for ratiometric sensors. The latter sensors are advantageous, as the ratio corrects for changes in cell volume, movement and sensor concentration [199]. For further
Simultaneous detection of $G_{\alpha q}$ and $(Ca^{2+})_i$ covariation

studies, it will therefore be beneficial to use ratiometric red fluorescent sensors such as Fura-2, an indicator we could not use with our equipment.

Intracellular calcium is a well-studied and often-used read-out of GPCR signaling; several approaches with different characteristics are available which make it easy to monitor. However, the signaling pathways leading to $(Ca^{2+})_i$ mobilization are diverse and intertwined [182] and can be influenced by various factors. Thus, intracellular calcium is rather a signaling network read-out than a specific receptor read-out and its dynamics have to be related to the cellular and experimental context in which they have been acquired. Further, there are different approaches to convert the oscillations usually observed in $(Ca^{2+})_i$ mobilization into dose-response curves. For example, the peak height, mean value or area under the curve are used to quantify its dynamics and impede the comparison of different studies.

Consequently, studies focusing on the biased signaling of a receptor can be improved by not solely relying on intracellular calcium as single read-out, but by combining two read-outs located at different layers within the network. By doing so, information about the contribution of the receptor to the bias as well as the amplifying or modulating influence of the signaling network for the propagation of the bias can be obtained. With this work, we showed that such an approach is feasible.

We find that ligand bias can be established at the onset of receptor signaling by receptor-G protein interactions and further be modulated by the downstream signaling network. We were able to trace the cellular responses to different ligands and monitor the signaling dynamics in single cells. Large cell-to-cell variations have been found in cell populations with the same origin [180, 181], and also in our experiments we found differences between the single cells upon the treatments. Our results therefore show that single-cell based pharmacology adds information to the existing high-throughput analyses. It shows that in cases where the cells show qualitative different dynamics, even subpopulations of cells should be distinguished.

Studies tackling the onset and the propagation of ligand bias in single living cells, for instance by using multicolor fluorescent microscopy in real-time, therefore have great potential to improve our understanding of ligand bias and might turn useful in the directed design of biasing drugs.

4.4 Materials and Methods

Materials

Dulbecco’s Modified Eagle Medium (DMEM) + GlutaMax, calcium free DMEM, Hank’s Balanced Salt Solution (HBSS), penicillin-streptomycin (pen/strep), Lipofectamine 2000 transfection reagent and Hygromycin B were purchased from Life
Technologies; the allosteric CaSR modulators R-568 and NPS-2143 from Tocris Biosciences. All other chemicals were from Sigma-Aldrich if not stated otherwise. HEK-293 wild-type cells were obtained from the American Tissue Culture Collection (ATCC® CRL-1573™). HEK-293 cells stably expressing the human calcium-sensing receptor (CaSR-HEK) were a friendly gift from Dr. Donald Ward, The University of Manchester. The plasmid containing the cDNA encoding human $G_{\alpha q}$, $G_{\beta 1}$ and $G_{\gamma 2}$ tagged to the fluorescent proteins are described in [169, 170]. Plasmids encoding for R-GECO-1 were obtained from addgene (www.addgene.org, CMV-NLS-R-GECO, plasmid #32462).

**Cell Culture & Transfection**

HEK-293 cells and CaSR-HEK cells were grown in DMEM + GlutaMax supplied with 10% FCS and 1 % penicilin/streptomycin. In addition, CaSR-HEK cells were under Hygromycin B selection (200 μM/ml). Two days prior to the experiment, the cells were seeded on fibronectin-coated glass coverlips. Lipofectamine 2000 was used according to the manufacturer’s instructions to doubly transfect the cells with plasmids encoding the FRET sensor $G_{\alpha q}$-CFP / $G_{\beta \gamma}$-YFP and R-GECO-1 the day before the experiment.

**Wide-Field Fluorescent Microscopy**

HEK-293 cells and CaSR-HEK cells grown on coverslips were mounted into an Attofluor cell chamber and maintained in microscopy medium (20 mM HEPES (pH = 7.4), 137 mM NaCl, 5.4 mM KCl, 0.5 mM CaCl$_2$, 0.8 mM MgCl$_2$ and 20 mM glucose). They were placed on a Zeiss inverted microscope (Axiovert 200M) equipped with an oil immersion 40x objective and kept at 37°C. For simultaneous measurements of CFP, YFP and R-GECO-1 emission, the cells were excited at 420 nm (CFP excitation) and additionally excited at 570 nm (RFP excitation). The CFP/YFP emission wavelengths were detected with a 470/30 and 535/30 bandpass filter by automatically turning the filter wheel. A 620/60 bandpass filter was used to detect R-GECO-1 emission. This was accomplished by automatically changing the filter blocks. The images were binned (4x4) and recorded with a cooled charged coupled device camera (Coolsnap HQ, Roper Scientific, Tucson, AZ, USA). The illumination time was set to 210 ms for both CFP and YFP. The illumination time for R-GECO-1 was set to 100 ms.

**Experimental Design**

Prior to the experiments, the cells were Ca$^{2+}$-depleted to reduce G protein activity induced by the CaSR to a minimum. The medium of the cells was changed twice with serum free DMEM containing 0.5 mM Ca$^{2+}$ and subsequently incubated in the
Simultaneous detection of $G_\alpha_q$ and $(Ca^{2+})_i$ covariation

low-calcium medium for at least 20 min prior to experiments at 37°C and 5% CO₂.
The cells were stimulated with stepwise increasing agonist concentrations every 90
seconds to allow the formation of a new steady state of $G_q$ activity and new levels
of intracellular calcium concentrations. Direct agonists used were Ca\(^{2+}\) and Sr\(^{2+}\).
Dependent on the experimental set up, CaSR specific drugs and modulators were
added to the cells 90 seconds before the Ca\(^{2+}\) stimulation as follows: 1 μM allosteric
inhibitor NPS-2143 or 1 μM calcimimetic R-568, or 10 mM Phenylalanine NPS-2143
and R-568 were dissolved in DMSO. It was made sure that only a total amount of 0.01
% DMSO was added to the cells.

**Image and data analysis**

ImageJ (http://rsbweb.nih.gov/ij/) has been used to analyze the imaging data: The
average fluorescence intensity of individual cells in the CFP, YFP and RFP channel in-
tensity were obtained by drawing regions of interest (ROI) of the individual cells.
Background intensity was obtained by selecting an ROI outside of cells. The back-
ground data was subtracted from average intensities of the cells and the data was
normalized to the average of the first 20 frames without stimulus. Further, YFP data
was corrected for crosstalk from CFP emission into the YFP channel. These normal-
ized intensity data were used to calculate the YFP/CFP ratio, which accounted as a
measure of $G_\alpha_q$ activity. RFP data was normalized as described above.

Mean intensities of CFP, YFP and YFP/CFP ratio data as well as RFP (R-GECO-1)
fluorescence and standard error of the mean (SEM) were calculated from at least 25
cells, obtained in at least 12 independent experiments. For the dose response data,
short periods of the steady states intermediating the stimulus additions have been
chosen for which the YFP/CFP ratio is plotted against the corresponding agonist dose.
Same holds true for the RFP fluorescence emission, whereas steady states were hardly
obtained in these measurements and mean intensities were used for the dose-response
curves. YFP/CFP ratios have been fitted to a distorted Hill equation (equation 4.1)

$$y_{model} = \alpha + \frac{\beta x^h}{K^h + x^h}$$  \hspace{1cm} (4.1)

for which $x$ being the agonist concentration and $y$ the normalized fluorescent intensity.
$K$ and $h$ are the dissociation constant and Hill coefficient, respectively with $\alpha$ as the
basal value and $\beta$ the distortion factor. Fitting of the complete data sets was conducted
by minimizing the objective function, the weighted sum of squares residuals (equation
4.2):
\[
obj_{\text{complete}} = \sum_{i=1}^{n} \frac{(y_{\text{model},i} - y_{\text{data},i})^2}{\sigma_{\text{data},i}^2},
\]

(4.2)

Here, \(y_{\text{model}}\) denotes the fluorescent intensity calculated by the model, described in equation 4.1; \(y_{\text{data}}\) are the data points for agonist concentration \(i\). \(\sigma_{\text{data},i}\) is representing the standard deviation of the data per concentration \(i\).

In order to correct for the decrease in fluorescence of R-GECO-1 over time (bleaching), the time course of R-GECO-1 emission (Fig. 4.3B) was fitted to an exponential decay curve:

\[
y = aE^{-(k\cdot t)^\gamma}.
\]

(4.3)

Values for the parameters were found as follows: \(a = 1.3, k = 0.14, \gamma = 0.23\) and R-GECO-1 data was corrected for the bleaching in all experiments. All corrections and analyses, as well as plotting and curve fitting have been carried out using Wolfram Mathematica version 10.0.
Simultaneous detection of $G_{aq}$ and $(Ca^{2+})_{i}$ covariation
Figure 4.9: Calcium titration experiment in double transfected cells. CaSR-HEK cells transiently transfected with the Gq FRET sensor and R-GECO-1 were kept in low calcium medium (0.5 mM) for 20 minutes before the experiment. During the experiment, the cells were exposed to cumulative increasing calcium concentrations of 0.5, 1, 2, 3, 4, 5 and 7 mM and CFP, YFP and RFP emission wavelengths were recorded. The time-lapse between stimulus addition was 90 seconds. 10 µM ionomycin were added 90 seconds before the end of the experiment. n = 42 cells. A) time traces of CFP (upper) and YFP (middle) emission and YFP/CFP ratio (lower panel) of the individual cells. B) Mean traces of the time course data for CFP (cyan), YFP (yellow) and YFP/CFP ratio (red). C) Dose-response curves generated from the steady-state responses of CFP (upper), YFP (middle) and YFP/CFP ratio (lower panel) to the increasing stimulus concentrations. D) Time traces of R-GECO-1 (RFP) emission of the individual cells (colored lines) and mean traces (black). E) Dose-response curves generated from responses of R-GECO-1. In all plots, error bars indicate SEM; in the dose-response curves the empty circles represent individual cells, the filled circles represent mean values. Solid lines in the dose-response curves were fitted to equation 4.1.
Simultaneous detection of $G_{q}$ and $(\text{Ca}^{2+})_{i}$ covariation

**Figure 4.10: Strontium titration experiment in double transfected cells.** CaSR-HEK cells transiently transfected with the $G_{q}$ FRET sensor and R-GECO-1 were kept in low calcium medium (0.5 mM) for 20 minutes before the experiment. During the experiment, the cells were exposed to cumulative increasing strontium concentrations of 0, 1, 3, 5, 7, 9 and 11 mM and CFP, YFP and RFP emission wavelengths were recorded. The time-lapse between stimulus addition was 90 seconds. 10 µM ionomycin were added 90 seconds before the end of the experiment. n = 25 cells. A) time traces of CFP (upper) and YFP (middle) emission and YFP/CFP ratio (lower panel) of the individual cells. B) Mean traces of the time course data for CFP (cyan), YFP (yellow) and YFP/CFP ratio (red). C) Dose-response curves generated from the steady-state responses of CFP (upper), YFP (middle) and YFP/CFP ratio (lower panel) to the increasing stimulus concentrations. D) Time traces of R-GECO-1 (RFP) emission of the individual cells (colored lines) and mean traces (black). E) Dose-response curves generated from responses of R-GECO-1. In all plots, error bars indicate SEM; in the dose-response curves the empty circles represent individual cells, the filled circles represent mean values. Solid lines in the dose-response curves were fitted to equation 4.1.
Ca\textsuperscript{2+} titration + 10 mM PHE

\(G_q\) FRET-sensor and R-GECO-1 transfection

Figure 4.11: Calcium titration experiment in the presence of 10 mM phenylalanine in double transfected cells. CaSR-HEK cells transiently transfected with the \(G_q\) FRET sensor and the intracellular calcium indicator R-GECO-1 were kept in low calcium medium (0.5 mM) for 20 minutes before the experiment. 90 seconds prior to the calcium additions, 10 mM PHE were added to the medium and kept constant. During the experiment, the cells were exposed to cumulative increasing calcium concentrations of 0.5, 1, 2, 3, 4, 5, and 7 mM and CFP, YFP and RFP emission wavelengths were recorded. The time-lapse between stimulus addition was 90 seconds. 10 \(\mu\)M ionomycin were added 90 seconds before the end of the experiment. n = 28 cells. A) time traces of CFP (upper) and YFP (middle) emission and YFP/CFP ratio (lower panel) of the individual cells. B) Mean traces of the time course data for CFP (cyan), YFP (yellow) and YFP/CFP ratio (red). C) Dose-response curves generated from the steady-state responses of CFP (upper), YFP (middle) and YFP/CFP ratio (lower panel) to the increasing stimulus concentrations. D) Time traces of R-GECO-1 (RFP) emission of the individual cells (colored lines) and mean traces (black). E) Dose-response curves generated from responses of R-GECO-1. In all plots, error bars indicate SEM; in the dose-response curves the empty circles represent individual cells, the filled circles represent mean values. Solid lines in the dose-response curves were fitted to equation 4.1.
Figure 4.12: Calcium titration experiment in the presence of calcilytic in double transfected cells. CaSR-HEK cells transiently transfected with the Gq FRET sensor and the intracellular calcium indicator R-GECO-1 were kept in low calcium medium (0.5 mM) for 20 minutes before the experiment. 90 seconds prior to the calcium additions, 1 μM NPS-2143 was added to the medium and kept constant. During the experiment, the cells were exposed to cumulative increasing calcium concentrations of 0.5, 1, 2, 3, 4, 5, and 7 mM and CFP, YFP and RFP emission wavelengths were recorded. The time-lapse between stimulus addition was 90 seconds. 10 μM ionomycin were added 90 seconds before the end of the experiments. n = 44 cells. A) time traces of CFP (upper) and YFP (middle) emission and YFP/CFP ratio (lower panel) of the individual cells. B) Mean traces of the time course data for CFP (cyan), YFP (yellow) and YFP/CFP ratio (red). C) Dose-response curves generated from the steady-state responses of CFP (upper), YFP (middle) and YFP/CFP ratio (lower panel) to the increasing stimulus concentrations. D) Time traces of R-GECO-1 (RFP) emission of the individual cells (colored lines) and mean traces (black). E) Dose-response curves generated from responses of R-GECO-1. In all plots, error bars indicate SEM; in the dose-response curves the empty circles represent individual cells, the filled circles represent mean values. Solid lines in the dose-response curves were fitted to equation 4.1.
Figure 4.13: Calcium titration experiment in the presence of calcimimetic in double transfected cells. CaSR-HEK cells transiently transfected with the $G_q$ FRET sensor and the intracellular calcium indicator R-GECO-1 were kept in low calcium medium (0.5 mM) for 20 minutes before the experiment. 90 seconds prior to the calcium additions, 1 μM NPS-R-568 was added to the medium and kept constant. During the experiment, the cells were exposed to cumulative increasing calcium concentrations of 0.5, 1, 2, 3, 4, 5, and 7 mM and CFP, YFP and RFP emission wavelengths were recorded. The period between stimulus addition was 90 seconds. 10 μM ionomycin were added 90 seconds before the end of the experiment. n = 49 cells. A) time traces of CFP (upper) and YFP (middle) emission and YFP/CFP ratio (lower panel) of the individual cells. B) Mean traces of the time course data for CFP (cyan), YFP (yellow) and YFP/CFP ratio (red). C) Dose-response curves generated from the steady-state responses of CFP (upper), YFP (middle) and YFP/CFP ratio (lower panel) to the increasing stimulus concentrations. D) Time traces of R-GECO-1 (RFP) emission of the individual cells (colored lines) and mean traces (black). E) Dose-response curves generated from responses of R-GECO-1. In all plots, error bars indicate SEM; in the dose-response curves the empty circles represent individual cells, the filled circles represent mean values. Solid lines in the dose-response curves were fitted to equation 4.1.
Simultaneous detection of $G_{\alpha_{q}}$ and $(\text{Ca}^{2+})_{i}$ covariation

Figure 4.14: Comparison of transfection with the $G_{\alpha_{q}}$ FRET-sensor and double transfection with $G_{\alpha_{q}}$ FRET-sensor and R-GECO-1. CaSR-HEK cells were either transfected solely with the $G_{\alpha_{q}}$ FRET-sensor (A), or doubly transfected with R-GECO-1 and the $G_{\alpha_{q}}$ FRET-sensor (B). In the presence or absence of allosteric modulators, CaSR ligands were increased in a step-wise manner as described above, and fluorescence emission of CFP and YFP was recorded. Despite different absolute numbers and slightly different kinetics, the results from the two different transfections show a qualitatively comparable behaviour. Here, the mean data of at least 16 cells are depicted, error bars indicate SEM. The solid line depicts the fit to equation 4.1.
G protein-coupled receptor signaling networks from a systems perspective

In collaboration with:
Boris N. Kholodenko, Martine J. Smit, and Frank J. Bruggeman

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Abstract

The signal-transduction network of a mammalian cell integrates internal and external cues in order to initiate adaptive responses. Amongst the cell-surface receptors are the G protein-coupled receptors (GPCRs) that have remarkable signal-integrating capabilities. Binding of extracellular signals stabilizes intracellular-domain conformations that selectively activate intracellular proteins. Hereby, multiple signaling routes are activated simultaneously to degrees that are signal-combination dependent. Systems-biology studies indicate that signaling networks have emergent processing capabilities that go far beyond those of single proteins. Such networks are spatiotemporally organized and capable of gradual, oscillatory, all-or-none and subpopulation-generating responses. Protein-protein interactions, generating feedback and feedforward circuitry, are generally required for these spatiotemporal phenomena. Understanding of information processing by signaling networks therefore requires network theories in addition to biochemical and biophysical concepts. Here we review some of the key signaling-systems behaviors that have been discovered recurrently across signaling networks. We emphasize the role of GPCRs, so far under appreciated receptors in systems-biology research.

5.1 Systems biology of signaling networks

We are far from a complete molecular understanding of how a single mammalian-cell makes its decisions, given extracellular and intracellular signals. We do not yet have the capabilities to determine the complete connection topology of its signaling networks (although we are moving towards this goal [200, 201]), how it adapts in time, and how the kinetic properties and interaction partners of the signaling proteins shape network responses. Such information is required for truly predictive medicine that is expected to rely heavily on mathematical models [202–204].

Great strides have however been made in identifying key principles of cellular signaling, at the level of single proteins and protein networks, including spatiotemporal aspects [205, 206]. These efforts promise to lead to a set of standardized, quantitative approaches for mapping and exploring the dynamic-signaling capabilities of protein networks. That such an approach can be a great success is indicated by current molecular and cellular biology. Generic protein concepts, such as, for instance, cooperativity, allostery and catalysis, have led to a standardized nomenclature and design of experiments that greatly accelerates molecular-biology approaches. The subsequent combination of molecular concepts with network concepts by systems biology is similarly powerful. For metabolism this already had great consequences. All metabolic capabilities of a cell can nowadays be computed with metabolic-network models [207]. Cell biology and systems biology are together aiming at reaching a similar status for the signaling network of a cell, requiring the merger of network con-
cepts with the biochemistry of signaling proteins [200, 201]. This is for many reasons, however, a much harder problem.*

5.2 Why GPCRs are so attractive for systems biology

From a biophysics and systems biology perspective, G protein-coupled receptors (GPCRs) are particularly attractive. They can attain different conformations that co-exist in a thermodynamic equilibrium, which shifts under the influence of signal binding [208]. Agonist binding will induce a conformational change of the receptor that activates intracellular signaling, while inverse agonists inhibit basal signaling and force the receptor in an inactive state. Allosteric modulators, which bind to allosteric sites of the GPCR, promote conformational changes that can alter orthosteric ligand affinity and/or efficacy, or may selectively activate a specific signaling pathway. For long it was thought that GPCRs exclusively signal via G proteins. However, GPCRs can also signal via G protein-independent signaling pathways that involve, for example, G protein receptor kinases (GRKs) and β-arrestins or GPCR interacting proteins (GIPs) that activate (unexplored) G protein-independent signaling pathways. Intriguingly, different ligands that bind to the same GPCR protein are able to induce distinct downstream signaling pathways. These biased ligands selectively stabilize only a subset of receptor conformations, thus preferentially modulating certain signaling pathways. Classical approaches for equilibrium-binding models of cooperative proteins immediately apply to GPCRs [209, 210] and can account for signal integration by receptors – so called ‘combinatorial ligand-bias’ [69]. This is in contrast to, for instance, receptor tyrosine kinases (RTKs) that require phosphorylations, a kinetic non-equilibrium process, for activation and conformation transitions.

The activation of G-proteins by GPCRs and their inactivation by GTPases, leading to steady-state activation of G-proteins, can be understood to a great extent in terms of existing principles of covalent-modification cycles [211–213]. Likewise, biochemical equilibrium-models of competitive binding describe the competition of immediate downstream proteins, such as G-proteins, GPCR receptor kinases (GRKs), and β-arrestins, for receptor conformations.

The conceptual and quantitative framework for understanding GPCRs and G-protein activation is therefore largely in existence, shifting the challenges more to the experimental and data-analysis side. Yet, systems biology has focussed much more on RTK-induced cellular signaling (the RAF-MEK-ERK axis) than on GPCR-activated networks.

*The calculation of the metabolic capabilities of a cell requires only the knowledge of the stoichiometry of the metabolic reactions encoded on the cell’s genome, not their kinetics. This is not so for the signaling capabilities of a cell, which do require the kinetics of the proteins, especially since signaling is such a dynamic phenomenon. Kinetic assays with signaling proteins are however notoriously hard. Whereas kinetic constants for metabolic enzymes can be determined in cell-free extract, this is not feasible for many signaling proteins, in particular for those that are active in protein complexes and the cellular membrane.
In this review, we aim to introduce the GPCR researchers to several systems-biology findings about signaling networks. This review is organized in a pragmatic manner (figure 5.1), we start by reviewing some of the basic biophysics aspects of GPCRs, followed by the kinetics of G-protein activation cycles, and finally we discuss several of the dynamic phenomena emerging in signaling networks that have been found with systems-biology approaches. Throughout this review we placed informative boxes, in which we explain a finding in quantitative systems-biological or biophysical terms, using mathematical models. We hope that this review sparks further interest in systems-biology studies of the principles of GPCR signaling networks.

Box 1: Membrane-diffusion and the time scales of dimerization equilibria of receptors. GPCRs are very dynamic, they continuously diffuse through the plasma membrane and dimers (or oligomers) are continuously forming and falling apart. Dynamic dimerization equilibria exist that are characterized by a constant, rapidly-fluctuating monomer and dimer fraction. Kasai et al [21] have recently quantified the monomer-dimer equilibrium of the N-formyl peptide receptor (FPR), a GPCR. They found that:

\[
2 \frac{m^-}{m^+} \rightleftharpoons m_2,
\]

\[k^+ = 1.085 \times 10^{-3} \text{ copies cell}^{-1} \text{s}^{-1}, \quad k^- = 11 \text{ s}^{-1}, \quad K_D = \frac{k^-}{k^+} = 10138 \text{ copies cell}^{-1},
\]

indicating that the dimer, denoted by \(m_2\), life-time is about 0.1 seconds (1/k^-): 10 dimers fall apart every second. The total number of receptor monomers per cell, \(m_T\), was 6000. At thermodynamic equilibrium, when \(K_D = \frac{m^2}{m_2}\), and given \(m_T = m + 2m_2\), the equilibrium concentrations of the monomer and the dimer become: \(m = 3534 \text{ copies cell}^{-1}\) and \(m_2 = 1233 \text{ copies cell}^{-1}\): less than 50% of the monomers exist as dimers. The life time of a monomer equals 0.4 seconds \(= \left( \frac{k^+ m^2}{m_T} \right)^{-1}\). The diffusion coefficient of a monomer in the membrane sets the upper limit of the association rate constant [214] to \(3 \times 10^{-3} \text{ cell}^{-1} \text{s}^{-1} \) [215]. The \(k^+\) of FPR is very close to this limit. Given a realistic diffusion coefficient (\(D\)) of 0.1 \(\mu m^2\) s for a GPCR [216], the average distance that a GPCR travels in a membrane in one second equals \(\sqrt{4Dt} = 0.6 \mu m\).
Figure 5.1: Overview of receptor and network modules that are involved in cellular decision-making and that are discussed in this review. Dimerization kinetics and ligand bias are biophysical aspects of GPCRs. The kinetics of G-protein activation cycles and of covalent-modification cascades together determine the sensitivity properties of signaling networks while feedbacks induce dynamic phenomena such as robustness, oscillations and bistability.

5.3 Quantification of the membrane processes

In systems biology, emphasis is put on quantitative studies [217]. In case of GPCRs, this concerns quantification of their kinetics and abundance. Quantification is, for instance, required for understanding the signaling outcome of competition of multiple
GPCRs for the same pool of G proteins [218]. This requires knowledge of ratios of receptor abundances and conformation-dependent G protein affinities. The expectation is that the kinetic constants of a GPCR will be constant across cell types, as long as isoenzymes or covalently-modified variants do not occur, while their abundances (expression level) can greatly vary. In addition, monomer-mobility and oligomerization affinities of GPCRs are key factors, as they determine the active fraction of receptors.

Box 2: Conformation equilibria of a GPCR and their shifts upon ligand binding.
We consider a receptor homodimer. Each monomer has an extracellular domain, with two ligand binding-sites. A homodimer has a single intracellular G-protein binding-domain. The homodimer is in an ‘off’ state when its subunits are both in their ‘T’ (tensed) conformation. When the subunits are in the ‘R’ (relaxed) conformation the receptor can activate G-proteins in an intracellular-domain-state specific manner. We consider two G-proteins, ‘1’ and ‘2’, that are activated by the receptor in the R state if its intracellular binding domain is in the matching ‘1’ or ‘2’ conformation. This is a minimal mechanism of conformation selection by ligand combinations and conformation-dependent G-protein activation. The fraction of active, ligand-saturated receptor with its intracellular domain in state ‘1’ is 

\[
\frac{r_1 s_4}{r_T} = \frac{\left( \frac{s^2}{a K_{R,1}} \right)^2}{(z_{R,1} + \ell_R z_{R,2})^2 + L (z_{T,1} + \ell_T z_{T,2})^2}.
\]

It depends on the ligand concentration \(s\), the conformation-dependent affinity of the subunits for the ligand \(K_{R,1}\), the allosteric factor \(a\), the intracellular-domain conformation equilibrium constants \(\ell_R\) and \(\ell_T\) and, finally, on the receptor equilibrium constant \(L\). The power ‘2’ indicated in red signifies the additional sensitivity of the receptor due to the fact that it is a dimer, the dimer makes the active fraction dependent on \(s\) raised to the power 4 (rather than 2). When positive allostery is really potent and the receptor its affinity for the ligand is independent of its intracellular domain conformation then,

\[
\frac{r_1 s_4}{r_T} = \frac{\left( \frac{s^2}{a K^2} \right)^2}{\left( 1 + \frac{s^2}{a K^2} \right) (1 + \ell_R) (1 + \ell_T)}.
\]

This equation gives a sigmoidal dependency of the active receptor fraction on the ligand concentration. The Hill coefficient of this equation equals \(2 \frac{\partial \ln r_1 s_4}{\partial \ln s}\) at \(s = s_{0.5}\) when \(r_1 s_4 = \frac{r_T}{2}\). This model displays all the features associated with ligand bias [69]. Ligand affinity, maximal response, and signal sensitivity are all dependent on the precise combination of ligands bound to the extracellular binding domain, in agreement with experiments.

Advanced techniques have been developed for the determination of receptor mobility and dimerization properties. In particular, tracking of single receptor-molecules at the surface of intact cells has proven very informative [99]. Receptors are expressed at several hundreds to several thousands of copies per cell. They are surprisingly
mobile with often localization-independent diffusivities [97, 216]. The dimerization kinetics for several members of distinct GPCR-families has been determined as well [21, 216, 219]. In box 1, we discuss the quantitative aspects of dimerization kinetics on the basis of recent findings.

5.4 Receptor activation and conformation selection

The advantage of receptor dimerization, and, in general, of oligomerization, is that the signal affinity can be better modulated, via allosteric interactions, and the signal sensitivity can be greatly increased, via cooperativity [210]. An influential view is that the functional, signaling states of the receptor correspond to specific receptor conformations, in particular of their intracellular domain to which downstream signaling proteins bind. The presence of extracellular ligands is transduced to the intracellular domain by ‘conformation selection’: the precise combination of ligands bound to the extracellular domain stabilises particular conformations of the intracellular domain. Whether this involves stabilization of multiple conformations of a single GPCR in particular fractions, leading to a ‘G-protein code’ that is ligand specific, or of a single conformation is unclear, and presumably even GPCR dependent.

These ideas are in flux at the moment, with the introduction of powerful structural methods. Structural studies provide evidence for conformation selection and conformation-dependent activation of downstream signaling proteins [220]. Kinetic information is given additional support for this mechanism [33, 99, 100, 221].

It is not a new concept in biochemistry that cooperative proteins can accommodate alternative conformations with different activities. Theories on cooperative proteins, reviewed by Changeux [210], have been describing such behavior already for a long time, early on mostly for metabolic enzymes and transcription factors. Allostery and cooperativity are key signaling features of GPCRs that make them versatile signal integrators.

An emerging view is that conformation selection by combinations of bound ligands and conformation-specific G-protein activation underlies ligand bias of GPCRs. This behavior can be accounted for by extended models of cooperative proteins [69]. In box 2 we present an example of such a model. Cooperative proteins are generally composed out of multiple subunits with allosteric interactions between ligand binding sites. Those properties play an important role in pharmacological applications, where conformation selection is used as a drug-design objective for altering ligand bias [222, 223].

Ligands can induce shifts in the conformation equilibrium of a GPCR, influencing its activation of intracellular G proteins and β arrestins [72] (see figures 5.1, 5.2). Using spectroscopy methods, Rahmeh and colleagues were able to show that distinct con-
formations of the V2 arginine-vasopressin (AVP) receptor were induced in response to different (biased) ligands [162]. The resulting ligand-biased signaling, or functional selectivity, is now viewed more often as an intrinsic property of the receptor-ligand complex, which can be understood from the thermodynamics associated with stabilization of particular receptor conformations [77]. Approaches for quantification of biasing effects has recently been reviewed [224]. During quantification of ligand bias, it is important to discern receptor biases from other sources, such as the signaling network [80].

In addition to classical, extracellular signals, peptides, pepducins and nanobodies that bind the receptor intracellularly can also affect the coupling GPCR to signaling molecules [225, 226] (reviewed in [86]). This indicates that the intracellular state can also modulate biased signaling. Scaffolding proteins, or other molecules that influence the receptor, may therefore prime the receptor from the inside for specific extracellular signals or alter intracellular signaling. A dynamic model of G-protein activation [227], which incorporates measured biochemical parameters, indicates the importance of cell-specific parameters, such as receptor and downstream protein expression ratios, and kinetic constants, for instance for GTP hydrolysis and ligand-binding affinities, for G-protein activation dynamics [227]. Similarly, a model of the M1 muscarinic receptor signaling, involving ligand-binding constants, receptor-G protein interactions and Gα-PLC interactions, gave rise to ligand-response relationships in close agreement with experiments [113]. The GPCR-field has advanced to a stage where enough kinetic information is available to quantitatively explain the dynamic responses of G-protein activation to extra- and intracellular stimuli. Such an approach is particularly relevant when mutant and wild type receptors are compared with respect to their signaling properties.

![Figure 5.2: Illustration of ligand bias and ultra sensitivity.](image)

_left: Two different signaling outputs are plotted against each other in a 'bias plot'. In such a plot, identical signaling responses lead to a straight line with slope 1 (solid line). Biased signaling responses causes a deviation from this linear relation and are represented by the curved, dashed lines._

_right: Ultra sensitivity is represented as a sigmoidal dose-response curve (solid line), whereas a hyperbolic curve, characteristic for a hyperbolic, ‘Michaelis-Menten’ response, is not ultra sensitive (dashed line).
Box 3: G-protein activation and sensitivity of its GTP-bound state ([G<sub>GTP</sub>]) to GPCR ligands

A GPCR acts as a guanine exchange factor (GEF) for G proteins. The resulting exchange of GDP by GTP leads to the activation of the G-protein; whereas a GAP hydrolyses this GTP,

\[
R^A + G^{GDP} \rightleftharpoons R^A G^{GDP} \rightleftharpoons R^A + G^{GTP} \tag{5.3}
\]

\[
GAP + G^{GTP} \rightleftharpoons GAP G^{GTP} \rightleftharpoons GAP + G^{GDP} \tag{5.4}
\]

Here \(R^A\) denotes the active conformation of the receptor. Using an equilibrium-binding model, these reactions lead to the following enzyme kinetic equation for the GEF and GAP activities,

\[
v_{\text{GEF}}(G^{GDP}, G^{GTP}) = k_{\text{GEF}} R^A \frac{G^{GDP}}{K_{\text{GEF}G^{GDP}}} \left[1 + \frac{G^{GDP}}{K_{\text{GEF}G^{GDP}}} + \frac{G^{GTP}}{K_{\text{GEF}G^{GTP}}} \right]^{-1}
\]

\[
v_{\text{GAP}}(G^{GDP}, G^{GTP}) = k_{\text{GAP}} \frac{G^{GTP}}{K_{\text{GAP}G^{GTP}}} \left[1 + \frac{G^{GDP}}{K_{\text{GAP}G^{GDP}}} + \frac{G^{GTP}}{K_{\text{GAP}G^{GTP}}} \right]^{-1}
\]

The sensitivities of the steady-state signaling output, the steady-state concentration of activated G-protein, \(G^{GTP}\), with respect to a change in the concentration of the GEF reaction catalyst, the active conformation of the receptor \((R^A)\) equals \([228]\),

\[
\frac{\partial \ln G^{GTP}}{\partial \ln R^A} = r_{G^{GTP}R^A} = \frac{[G^{GTP}] v_{\text{GEF}} G^{GDP} - v_{\text{GAP}} G^{GTP}}{[G^{GDP}] v_{\text{GEF}} G^{GDP} - v_{\text{GAP}} G^{GTP}} \tag{5.7}
\]

The \(\epsilon\) coefficients are generalized kinetic orders (or elasticity coefficients [228]) of the reactions: \(v = \frac{\partial \ln v}{\partial \ln [\text{substrate}]^0}\) and zero-order ultra-sensitivity occurs \([213]\). This is most noticeable, when we assume both enzyme rates insensitive to their product concentrations; then \(\frac{\partial \ln G^{GTP}}{\partial \ln R^A} = \frac{\epsilon_{G^{GTP}R^A} v_{\text{GEF}} G^{GDP} + \epsilon_{G^{GTP}R^A} v_{\text{GAP}} G^{GTP}}{\epsilon_{G^{GTP}R^A} v_{\text{GEF}} G^{GDP} + \epsilon_{G^{GTP}R^A} v_{\text{GAP}} G^{GTP}}\), which is much greater than 1 when the \(\epsilon\) coefficients are close to 0.

The sensitivity of \([G^{GTP}]\) to a ligand of the GPCR, \(R_S^{G^{GTP}} = \frac{\partial \ln G^{GTP}}{\partial \ln S}\), can be decomposed into \([228]\),

\[
R_S^{G^{GTP}} = \frac{\epsilon_{G^{GTP}R_A} v_{\text{GEF}} G^{GDP} + \epsilon_{G^{GTP}R_A} v_{\text{GAP}} G^{GTP}}{\epsilon_{G^{GTP}R_A} v_{\text{GEF}} G^{GDP} + \epsilon_{G^{GTP}R_A} v_{\text{GAP}} G^{GTP}} \tag{5.8}
\]

In a dose-response curve of \(\log[G^{GTP}]\) as function of \(\log S\), the slope, which is the sensitivity, equals the product of the receptor sensitivity, \(r_{S}^{G^{GTP}R_A}\), and the G protein sensitivity, \(r_{R_A}^{G^{GTP}}\). Therefore, ‘sensitivity amplification’ \([212, 228, 229]\) occurs when both \(r\)’s exceed 1.
5.5 G protein activation and inactivation cycles

Upon binding of a GDP-bound G-protein to an active conformation of a GPCR, the G protein can become activated in this complex via a guanine exchange event, this kinetic activity presumably resides on the GPCR [230]. The activating conformation change of the GPCR causes it to have GEF activity. Upon activation, the G-protein can dissociate from the complex and activate downstream signaling until it is inactivated by its GTPase activity usually enhanced by GTPase-activating proteins (GAPs). The G-protein therefore cycles between active and inactive states. The fraction of active G-protein is determined by the balanced between GEF and GAP activities [230]. In box 3 we show some of the basic aspects of such processes.

Covalent-modification cycles, e.g. formed by a kinase and phosphatase pair, can display zero-order ultra sensitivity with respect to signals (figure 5.2)[175, 213, 229]. A GPCR activation/inactivation cycle can in principle show the same behavior – even though it is not strictly a covalent-modification cycle – with respect to signals acting on the GPCR.

In case of ultra sensitivity, a large fractional change in the $G^{GTP}$ concentration occurs upon a small fractional change in the concentration of the ligand that binds to the receptor. Accordingly, the dose-response curve of $[G^{GTP}]$ as function of the signal concentration is switch-like (e.g. with a Hill coefficients exceeding 4). Switch-like, zero-order ultra-sensitive behavior occurs when both the GEF and the GAP enzymes are saturates with their substrates – $[G^{GDP}] >> K_{M}^{GEF}$ and $[G^{GTP}] >> K_{M}^{GAP}$ –, such that they operate in their zero order regime ($K_{M}$ denotes the Michaelis-Menten constant of the enzyme.) (box 3). Trunnel et al. [231] present an experimental illustration of ultra sensitivity.

Turcotte and coworkers [232] developed a kinetic model of a G-protein covalent-modification cycle and found ultrasensitivity in the calculated dose-response curves. Ultrasensitive responses have found experimentally with PIP3 activation [233]. In a combined experimental and modeling study, Karunarathne et al [233] demonstrated that PIP3 concentrations are ultrasensitive with respect to GPCR activation [233].

The phosphorylation of GPCRs by GRKs and the associated dephosphorylation events can also lead to G protein ultra sensitivity. GRKs have also been shown to have a broad influence on GPCR signaling [234]. Systems biology studies on GRK-regulated GPCR activity [114, 235] indicate the versatility GRK regulation and are examples of the insightful combinations of experiments and modeling.
Box 4: Sensitivity of networks to signals: cascades, feedback and feedforward networks

In a signaling cascade, the net sensitivity of the output $D$ with respect to the input $A$ at steady state equals the product of the sensitivities along the cascade [228, 236], with $A$-to-$D$ as concentrations of signaling proteins,

$$\frac{A}{r_A} \xrightarrow{r_B} \frac{B}{r_B} \xrightarrow{r_C} \frac{C}{r_C} \xrightarrow{r_D} D, \quad R_D^A = r_C r_B r_A (5.9)$$

All these local sensitivities, the $r$’s, can be obtained from steady-state dose-response curves. When $D$ feedbacks onto $A$, regardless of whether this is a positive or negative interaction, we obtain,

$$F_{R_D}^A = \frac{R_D^A}{1 - r_A^D R_D^A}, (5.10)$$

with $r_D^A$ as the feedback strength, it is $< 0$ in case of negative feedback and $> 0$ in case of positive feedback. The effect of negative feedback is that the sensitivity of the output $D$ with respect to the input signal $A$ is reduced. These equations loose their meaning when the feedback destabilises the system. In the case of positive feedback, stability is guaranteed as long as the denominator remains positive. The positive feedback sensitises the cascade for the signal when $F_{R_D}^A > R_D^A$. When $r_A^D R_D^A = 1$, the sensitivity becomes infinite and all-or-none response occurs – associated with ‘bistability’ –, which is a known response of signaling cascades. When feedbacks destabilise the system oscillations or bistability can occur as discussed in the main text.

5.6 Signal transduction cascades of biochemical reaction cycles: sensitivity amplification

Downstream of GPCRs and their direct activation targets, such as G-proteins and arrestins, signals that initiated at the cell surface are transduced via cascades of covalent-modification cycli, amongst other processes. The best understood cascade of covalent-modification cycles is the mitogen-activated protein kinase (MAPK) cascade, involving three covalent-modification processes in sequence [237]. GPCRs activate the ERK, JNK and p38 MAPK cascades using spatially and temporally distinct pathways that are dependent on either G-proteins or $\beta$-arrestins [238]. Direct MAPK activation via $G_\alpha$ is transient, $\beta$-arrestin independent, and can involve PKC activity [239]. The other pathway leading to MAPK activation is triggered by the recruitment of $\beta$-arrestins as scaffolding molecules [239, 240]. For $\beta$-arrestin-mediated ERK signaling, $\beta$-arrestins scaffold all three MAPK cascade kinases, Raf, MEK and ERK [241]. Since $\beta$-arrestins interact with components of the clathrin-mediated endocytic pathway, GPCRs bound to $\beta$-arrestins are targeted to clathrin-coated pits. For different GPCRs, $\beta$-arrestins either rapidly dissociate and receptors recycle to the plasma membrane, or GPCR-$\beta$-arrestin complexes stay together on the surface of endocytic vesicles. In either case, $\beta$-arrestins scaffolds mediate activation of multiple signaling proteins, (such
as the tyrosine kinase Src and MAPK cascades), thereby displaying a novel role of signal transducers in addition to their classic function of GPCR signaling attenuation [242]. Strikingly, only ERK molecules that are stimulated via G protein-dependent pathways translocate into the nucleus, leading to transcriptional activation and DNA synthesis, whereas active ERK molecules generated via β-arrestins accumulated on endosomal vesicles, are entirely retained in the cytoplasm [243]. Not only are the MAPK cascades that are activated via G proteins or β-arrestins spatially separated, but also their temporal activation profiles are remarkably different. In human embryonic kidney (HEK)-293 cells, G protein-dependent ERK1/2 activation is transient, rapidly reaching a peak at about 2 min and descending to low levels after about 10 min following stimulation, whereas activation via β-arrestins is sustained until at least 90 min [243]. Different spatiotemporal patterns of ERK stimulation via G proteins or β-arrestins entail distinct functional outcomes at the level of downstream ERK substrates. A decrease in G protein-dependent ERK stimulation due to increased expression of β-arrestins inhibits ERK-mediated phosphorylation of transcription factors, such as ELK-1, whereas β-arrestin mediated ERK signaling induces cell motility phenotype, which is associated with the rearrangement of cytoskeleton, membrane ruffling and chemotaxis, caused by prolonged activation and retention of active ERK in the cytoplasm.

Figure 5.3: Sensitivity amplification by a signal-transduction cascade. A covalent-modification cascade (left) can amplify the signal sensitivity (right). The signaling response becomes steeper along the signaling cascade.
The same principle of $\beta$-arrestin scaffolding leads to GPCR induced NF$\kappa$B signaling [244], a signaling cascade that is also controlled by a series of post-translational modifications. The MAPK system has been studied extensively and several interesting signaling features have been found, such as oscillations, drug-robustness and ultra sensitivity [245–248].

One finding is that the outputs of cascades of covalent-modification cycli can display a sensitivity to cascade input-signals that exceeds the sensitivity of any of its elemental cycli [228, 229, 246, 249]. For instance, the MAPK pathway of Xenopus oocytes was found to have a Hill coefficient of 8, which is truly a switch-like response [246], and believed to be due to ‘sensitivity amplification’. Sensitivity amplification can be understood in terms of intuitive theory [228], an example of this is shown in box 4, and with mathematical models based on enzyme kinetics [249]. How heightened sensitivity arises in cascades follows from basic principles of the enzyme kinetics of the associated kinases and phosphates [175, 213]. The cascade sensitivity equals the product of the sensitivities of the individual covalent-modification cycli in the cascade (box 4) (figure 5.3) [228, 236]. The sensitivity of a single covalent-modification cyclus depends on the extent of the saturation of the kinases and phosphatases (box 3).

Activation of signaling proteins by double-phoshoxylation, such as of MEK and ERK leading to $MEKP_2$ and $ERKP_2$, can cause to heightened sensitivity at the level of a single cascade element [250]. It has also been show to give rise to a more surprising phenomenon, called ‘bistability’, which is an all-or-none response of the output, e.g. of $ERKP_2$, with respect to the input, e.g. $MEKP_2$, with hysteresis properties. Bistability is explained in figure 5.5 and has been found in mathematical models for a number of signaling networks [250–253]. Bistability is also a mechanism that can lead to the formation of subpopulations in monoclonal cell cultures and it is for this reason associated with differentiation of stem cells [254, 255]. Ultra-sensitivity, sensitivity amplification and bistability [175, 256] are examples of network properties that arise from the interactions between protein activities and cannot be attributed to single proteins. Because multiple proteins are required for these systems properties, in addition to precise values of kinetic parameters and expression levels, mathematical models are useful tools when studying them [217].
Box 5: Drug-insensitivity of signaling cascades due to negative feedback. The response equation of a signaling network with negative feedback \[228, 236\],

\[ \frac{F^D}{R^D_A} = \frac{R^D}{1 - r^A_D R^D_A}, \]  

(5.11)

was introduced in the previous box. When negative feedback occurs, the feedback term \( r^A_D \) is negative. When \( -r^A_D R^D_A >> 1 \), which can occur when \( -r^A_D >> 1 \) or \( R^D_A >> 1 \), the previous equation can be approximated by,

\[ \frac{F^D}{R^D_A} \approx \frac{1}{-r^A_D}. \]  

(5.12)

This equation represents the slope of the dose-response curve of log \( D \) as function of log \( A \). This equation indicates that the response of the output \( D \) of a cascade with negative feedback to a change in its input \( A \) only depends on the feedback strength, provided the above mentioned conditions are met! This result has remarkable consequences. Firstly, it shows how cells can make their responses to signals insensitive to undesired disturbances that happen in their own signaling networks and affect \( R^D_A \). Secondly, the medical consequence is that the effect of drugs, acting on the signaling cascade and changing \( R^D_A \), will not have a huge effect as long as \( -r^A_D R^D_A >> 1 \); in particular in the case of strong negative feedback, when \( r^A_D >> 1 \) \[247, 248\].

5.7 Negative feedback in signal transduction cascades: oscillations and robustness to drugs

The output of signaling networks, such as cascades, often feedback to stimulate or inhibit the processing of the input(-s) of the network [257]. Negative feedback is a prerequisite for the emergence of oscillations. Feedback mechanisms arise from transcriptional regulation of cascade compounds, their covalent modification, endocrine actions or receptor initiation [258]. The study of negative feedbacks has revealed their impact on signaling and appear to contribute to adaptation, robustness and oscillations [204]. Oscillatory dynamics has been found in a range of signaling networks, e.g., during NFκB, ERK, and intracellular-Ca\(^{2+}\) signaling [259–262], many of which are linked to GPCRs. GPCRs are also directly regulated by negative feedback; for instance, via feedback activation of GRKs leading to inhibitory phosphorylation of a GPCR [263] or via other mechanisms [264].

Calcium oscillations are linked to GPCR signaling. Feedback inhibition by calcium of the agonist-receptor complex, presumably through the calcium-sensitive receptor kinases, has shown that oscillations of intracellular calcium are influenced by activation of \( G_\alpha \) [265] and the internalization rate of \( \beta \gamma \) subunits [266]. Various mathematical models exist that focus on different aspects of calcium oscillations (reviewed in [262]). Cross-talk between \( G_\alpha_i \) and \( G_\alpha_s \) regulated signaling, investigated with a modeling approach by Siso-Nadal et al. [267], suggests that both species can oscillate,
likely providing an additional layer of complexity that allows the cell to discriminate between different combinations of signals. Future studies with, for instance, G-protein FRET-sensors should give more insight into oscillations of G-protein activation. It was also found that GFP-tagged ERK displayed oscillations between cytosol and nucleus over a broad range of ligand concentrations [260]. Through modeling and experiments it was concluded that the negative feedback from ERK onto upstream proteins underlies these oscillations, which are likely functional during development [245, 260]. In NFκB signaling, a strong negative feedback of IκBα is leading to oscillations in NFκB [259]. Thus, the function of NF-kB as a transcription factor might therefore depend on the characteristics of its oscillations, leading to dynamic control of gene expression [261]. Direct involvement of negative feedback of GPCRs in signaling oscillations has, as far as we know, not yet been established.

Besides oscillations, robustness is another feature associated with negative-feedback activity. The negative feedback from ERK to upstream Raf in the MAPK pathway resembles a negative feedback amplifier (NFA), used in engineering to design robust systems [112]. The switch-like behavior of the RAF-MEK-ERK cascade, in the absence of feedback, is changed to a more gradual, linear response (illustrated in figure 5.4) by the feedback. The feedback causes robustness of phosphorylated ERK against variations in the total ERK level, via expression regulation, in agreement with theory and experiments [247]. It also contributed to robustness to a MEK inhibitor, during medical treatment [112, 247]. For colorectal cancer-cells it was shown that the negative feedback in EGFR signaling led to crosstalk between ERK and AKT signaling; inhibition of ERK resulted in activation of AKT, via EGFR, and combined inhibition of ERK and EGFR inhibited Ras, ERK and AKT [112, 268]. These predictions were first made with a mathematical model before being validated with a xenograft model. All these studies indicate that targeting of signaling networks at a single protein, for example during cancer treatments, can be insufficient and be overcome by the cell, due to compensatory feedback mechanisms. Likewise, studies indicate that the absence of a feedback, e.g. by a mutation in Raf, can sensitise cells to inhibitors [112, 247] (box 5). Mathematical models and network concepts are therefore providing new ways for studying signaling networks and predicting drug responses.
5.8 Positive feedback in signal transduction cascades: from gradual to all-or-none, bistable responses

While mild negative feedback makes cells robust and strong negative feedback causes them to oscillate, positive feedback increases signal-sensitivities, amplifies these sensitivities and can even lead to all-or-none, switch-like, binary responses [175, 204, 256]. Thus, negative and positive feedback have very different functions in a signaling network.

Positive feedback can cause networks to display bistability. Bistability has three features: a switch-like response, a history-dependent response (hysteresis) [175] and generation of cellular subpopulations (phenotypic diversification, cellular differentiation) [175, 255] (see figure 5.5). Bistability has been documented for a large number of systems. Most examples are currently of microbial systems, because the type of single-cell experiments required for identification of bistability are still laborious for mammalian cells. Bistability has however been shown in experiments of differentiating Xenopus oocytes [246, 269], during immune responses [270], cellular differentiation
[271, 272] and the MAPK pathway [246, 273]. A requirement for bistable behavior is that the signaling cascade displays sensitivity amplification along the feedback loop (box 4).

Theoretical analyses suggest that bistability can also arise in signaling networks via other mechanisms. For instance, double-phosphorylation of signaling proteins, such as of MEK and ERK in MAPK cascade, can bring about bistability even in the absence of feedback loops or cascade interactions [250]. Legewie and coworkers reported that complex formation in signaling cascades, for instance via scaffolds, can cause bistability [274]. One of the uses of mathematical models is therefore to explore the capabilities of signaling networks and then design targeted experiments that either validate or falsify model predictions.

Applications of quantitative, single-cell-based assays of signaling dynamics, e.g. using fluorescence methods, will undoubtedly lead to more cases of bistability and oscillations of signaling networks. With cell-population based methods those behaviors can generally not be observed.

Figure 5.5: Illustration of bistability. Positive feedback in a system can cause it become bistable. A bistable system can be in one out of two stable states, represented as two valleys in a hilly surface. The state is represented in this analogy by the position of the ball. A separating tipping-point – a metastable state – exists that separates the two stable states. When the system resides in the metastable state, fluctuations can cause the system to end up in stable state 1 or 2. Parameter changes, e.g. transcription rate, alter the shape of the surface, such that the number of stable states can vary from 1 to 2 to 1 (left figure). Depending on the direction of parameter changes (from low to high or the reverse), the system switches between stable states at different points. This phenomenon is called hysteresis.
5.9 Experimental observations of bistability and oscillations require single-cell approaches

The complication of studying dynamic features of signaling networks is that cell-population studies are not always reflective of the dynamic behavior of single cells. Since cells do generally not function in exact synchrony, oscillations may not be visible at the level of a population of cells, only at the single-cell level. Likewise, subpopulations that arise during bistability are not visible from cell population studies. Single-cell studies, using for instance fluorescent protein based methods, are therefore required to study dynamics of signaling circuits. For instance, studies of NFκB, intracellular Ca\(^{2+}\) and ERK oscillations and of bistability, during signaling and cellular differentiation, relied on fluorescent reporters [259–261, 275, 276].

Single-cell studies also indicate that isogenic cells, with the same cultivation history, display fluctuations in the level of signaling proteins, likely arising from stochastic events during transcription and cell division [181, 275–277], which can be understood from basic biophysics [278]. This phenomenon has been termed ‘noise’ [277]. Feedbacks in networks can both reduce and enhance noise. A consequence of noise is that cells can vary in their individual responses to physiological triggers [180], including drugs [181]. Kempe et al. [279] have quantified the variability of transcript level in human cells and showed that transcript-number variabilities between cells derive from differences in cell volume and stochasticity at the level of transcription. Noise is not only an undesirable feature of molecular systems that scrambles information transfer, it is functional as well. It is, for instance, intimately linked to bistability giving rise to subpopulations during cellular differentiation [280].

5.10 Fold-change detection by networks compensates for stochastic fluctuations in protein levels

Due to inevitable fluctuations in molecular activities [281, 282], cells exploit regulatory circuitry to reduce the harmful impacts of noise, occurring in signal transduction and transcription [283, 284]. In this way, cells increase the reliability of information transfer in signaling networks [285]. Negative feedback is an example of a noise-reduction mechanism [286].

Another mechanism for noise reduction is fold-change detection (box 6). Networks that detect fold changes have the same dynamics upon the same fold change in the stimulus, regardless of its basal level [287]; this is visualized in figure 5.6. An (incoherent) feedforward loop in a signaling network gives rise to fold-change detection [287], as well as other network motifs [288]. Fold-change detection has been reported for mammalian signaling [275, 287]. For instance, Cohen-Saidon and co-workers [275] studied the dynamics of nuclear ERK2 upon EGF stimulation using a
genetically-encoded, YFP-tagged ERK2 in single cells. They found that ERK levels in
the nucleus differed greatly from cell to cell, but the fold changes of individual cells,
upon EGF addition, was a much more homogeneous. In addition, the fold-changes
returned to basal levels after a transient period, indicating exact adaptation. All these
phenomena can be understood from a simple mathematical model of an
incoherent-feedforward loop network [275, 287].

Hart et al. [289] studied the integration of two different stimuli by fold-change
detecting systems, using mathematical models. They used Monod-Wyman-Changeux-
based models of receptors associated with bacterial chemotaxis. These models are
also applicable to GPCRs (box 2) and it is therefore tempting to speculate that some
GPCRs may have fold-change detecting properties as well.

These studies indicate the operation of some signaling systems should not be appreci-
atated in terms of absolute concentrations of signaling proteins, but rather in terms of
fold changes in concentrations of active signaling proteins. Again a system concept,
originating from engineering in this case, proves a useful concept for the quantitative
study of signaling networks.

Box 6: Fold change detection by a molecular activation cascade. A simple model of a
feedforward motif illustrates the principle of fold change detection [287]. We consider a system
with the signal $S$ and two signaling proteins $X$ and $Y$, their concentrations change according to,

\[ \begin{align*}
\frac{d}{dt} x &= k_1 s - k_2 x, \\
\frac{d}{dt} y &= k_3 \frac{s}{x} - k_4 y.
\end{align*} \tag{5.13} \]

$S$ therefore ‘feeds forward’, it directly activates $X$ and $Y$ and it activates $Y$ indirectly via $X$. At
steady state, when the concentration of the signal $S$ equals $s$, the concentration of $X$ and $Y$ are:

\[ x_s = \frac{k_1 s}{k_2}, \quad y_s = \frac{k_3 k_4 s}{k_1 k_2}. \]

Note that a change in $s$ does not affect $y_s$, $y$ therefore displays ‘exact
adaptation’; the influence that $s$ has on the synthesis rate of $Y$ is compensated for by $x$. To
show that this system is capable of fold-change detection, we normalise the concentration with
respect to their basal, steady-state levels at reference concentration $s$,

\[ E_x = \frac{x}{x_s}, \quad E_y = \frac{y}{y_s}, \quad E_s = \frac{s}{\bar{s}}, \tag{5.14} \]

with $\bar{s}$ as the new value of $s$, applied when the system is at a steady state at the basal value, $s$,
when $x = x_s$ and $y = y_s$. The dynamics of the fold changes in the concentration becomes,

\[ \begin{align*}
\frac{d}{d\tau} E_x &= E_s - E_x, \\
\frac{k_2}{k_4} \frac{d}{d\tau} E_y &= \frac{E_s}{E_x} - E_y
\end{align*} \tag{5.15} \]

with $\tau = k_2 t$. The fact that the concentration dynamics can be rewritten in terms of fold changes
indicates that the basal steady-state concentrations, at which the change in $s$ is applied, do
not matter. The same fold change in $s$ always give the same fold change in $x$ and $y$ regardless
of the basal levels $x_s$ and $y_s$. The system therefore responds to fold changes rather than to
absolute changes in concentrations. This system is therefore robust to undesired distorting changes in basal concentrations, as they do not affect signaling outcome as long as the outcome is considered normalized to the basal concentrations.

5.11 Outlook: appreciating how a GPCR plays a role in the decision making machinery of a cell

Presently, we understand the basic principles of how single GPCRs integrate signals and activate intracellular proteins in a biased, signal-combination dependent manner. Combination of new techniques, such as G-protein FRET-sensors, structural methods and mutant screens, will undoubtedly give deeper insights into single GPCR functioning in the near future.

Systems-biology studies of signaling networks are indicating that the signaling does not stop at the cell-surface receptors, much of the cellular signaling-capacities derive from those networks. Activation/inactivation cycles of proteins, signaling cascades and interwoven feedback and feedforward circuitry leads to counterintuitive, functional signaling-dynamics that is best understood with mathematical models and single-cell experiments. We therefore expect that the next developments in the GPCR-field will move towards quantitative single-cell studies on the signaling consequences of biased GPCR activation, using mathematical models for hypothesis generation and data integration. Such studies can elucidate the interplay between the receptors and the signal transduction machineries of the cell, this knowledge is of great value for medical applications (as e.g. proposed by [268]).

In this review, we gave a glimpse of results obtained by systems biology of cellular signaling. We discussed various network motifs of signaling pathways that have been investigated with respect to their potential effects on signal outputs. For example, we illustrated how ultra sensitivity arises and can result from cascade sensitivity-amplification. An important aspect was how feedbacks shapes signaling functions in a cell, including oscillations and robustness. We emphasise that many of these network-centered studies do not yet focus on GPCRs, even though those proteins are often the network inputs. Thus, we hope that future studies will investigate GPCR-signaling with systems approaches.

As more and more knowledge is gained about the crosstalk between membrane receptors and their induced signaling pathways [290], we are forced to envision signaling not as separated pathways, but as a network. A single receptor may then play only a minor role in setting the behavior of the whole cell, rather it may be that signal-integration by different receptors and intracellular wiring of protein-protein interactions is decisive. This view of signal transduction has great potential for the design and application of new (multi-target) therapeutics. The approach to
combine mathematical models and to validate their predictions with experimental data will enable new insights into the mechanisms of signal transduction and promises innovative clinical applications.

Figure 5.6: A network with an incoherent feed-forward loop can give rise to fold-change detection. Two identical fold-changes in the signal $X$, applied at two different of basal levels, give rise to basal-level dependent dynamics of $Y$, whereas the dynamics of $Z$ is basal-state independent (the dashed and full lines overlap). $Z$ therefore detects fold changes in $X$. 
CHAPTER 6

General Discussion
6.1 Multiplex signaling: is it the receptor, the network or both?

How are cells able to discriminate and integrate the multitude of signals that they receive via their membrane receptors? Is this mostly done by the receptor themselves? And, if so, how do receptors achieve signal integration (biased signaling)? Which biochemical mechanisms lead to these phenomena? Or is the downstream signaling network the major driver of signal integration, and do receptors only play a minor role? Only by directly measuring signaling bias introduced by a receptor and comparing this to the bias that is observed at downstream signaling we can answer those questions.

The central aim of this thesis is to investigate how and whether ligand bias occurs at the receptor level, at the onset of cellular signaling when a GPCR activates a heterotrimeric G protein, the first cytosolic signal transduction event. I investigated this research question using theoretical concepts and advanced fluorescence microscopy techniques.

I first studied a conformation-equilibrium model of GPCRs (chapter 2) to address the biochemical mechanism leading to receptor-level biased signaling. The model is an extension of the concerted symmetry model of cooperative proteins introduced by Monod, Wyman and Changeux. In our model, external signals induce two conformational changes. The first is a global conformation change, shifting the entire receptor from an inactive to an active state. The second conformation change is a local conformation change, involving conformation changes of the active state of the receptor; such that multiple active conformations can co-occur, can shift in proportions, and activate distinct intracellular signaling routes. The different active conformations are differentially stabilized by different ligands. The model allows for the calculation of the receptor fractions occurring in particular conformation states as function of the ligand concentrations and ligand combinations. Biased signaling occurs when extracellular ligands cause a shift in the ratios of active conformations. This is indeed what we found. We therefore show that biochemistry allows for the occurrence of ligand bias at the level of the receptor and that modifiers - for example, allosteric agonists - can modulate the receptor bias. Several studies indicate this kind of conformational flexibility of GPCRs [36, 70–73]. Improved structural methods, based on, for instance, crystallography, will further extend our knowledge about the influence of orthosteric and allosteric ligands on the receptor structure and its conformation equilibria[10, 291–293].

Additionally, I started experimental investigations. I focused on the human glutamate-like, extracellular calcium sensing receptor, the CaSR. This receptor has a surprisingly high capacity for multiplex signaling: it binds many external ligands, both at orthosteric and allosteric sites and it is coupled to multiple intracellular signaling pathways. CaSR is expressed in various tissues, performs different physiological functions and seems to play divergent roles in cancer development [50]. I
investigated the biasing effect of different agonists on CaSR-induced $G_{aq}$ activation (chapter 3). This study showed that time-resolved CaSR activity can be monitored by a $G_q$ FRET sensor in single cells. Thereby, we found similarities of the agonists’ kinetic properties described in the literature for downstream signaling [58, 59, 63, 168]. These similarities indirectly indicate a close coupling of $G_{aq}$ activity with calcium mobilization. We also observed differences in $G_{aq}$ activity after stimulation with polyamines compared to studies of calcium mobilization [174], indicating the amplifying role of the signaling network. Based on our findings, I conclude that ligand bias is partially established at the receptor level, and that the downstream signaling network can modulate signaling biases occurring at the onset of cellular signaling.

My findings on partial ligand bias of the receptor are further supported by my second set of experiments where I measured the simultaneous activation of $G_{aq}$ activity and the mobilization of intracellular calcium, $(Ca^{2+})_i$, upon CaSR activation (chapter 4). Those measurements show for the first time the co-activation of two CaSR signaling read-outs at different levels of the signaling cascade and in single cells. By taking the following perspective,

$$\text{signal(-s)} \xrightarrow{\text{CASR}} G_q \text{ activation} \xrightarrow{\text{downstream signaling}} (Ca^{2+})_i \text{ mobilization},$$

which I translate into a slightly more abstract, generally-applicable, picture,

$$\text{s} \xrightarrow{R(\cdot)} R(s) \xrightarrow{N(\cdot)} N(R(s)),$$

with ‘$R(\cdot)$’ and ‘$N(\cdot)$’ respectively denoting the receptor and network effects. Clearly, if the network output, $N$, always has the same value for the same value of $R$ then downstream signaling is signal independent and biased signaling caused by network effects does not occur. Plotting the $(Ca^{2+})_i$ vs $G_{aq}$ for different signal combination of the CaSR can therefore indicate the occurrence of network bias. If these plots vary with the signal combinations then network bias occurs. Indications of such a ligand-dependent variation have been observed in my co-activation study.

We expected to observe phenylalanine (PHE) as a signaling modifier of the $G_{aq}$ activity, as this impact of PHE has been already described on the basis of its effects on intracellular calcium oscillations [57, 65, 164, 165, 179]. We did not detect significant changes of $G_{aq}$ activity upon PHE administration, when compared to stimulation with $(Ca^{2+})_o$ alone (chapter 3). We did, however, observe effects of PHE on $(Ca^{2+})_i$ mobilization (chapter 4). Perhaps our method is not sensitive enough to detect minor changes at the G protein activation level. On the other hand, phosphorylations of T888 in the cytoplasmic tail of the CaSR by PKC are known to be key regulators of $(Ca^{2+})_i$ oscillations [63, 64]. Conformational changes of the CaSR induced by amino acid binding might therefore alter the binding affinity of the kinase and/or phosphatase and thereby additionally regulate $(Ca^{2+})_i$ dynamics, while G protein activation remains unaffected. These observations further reinforce the importance of the signaling network downstream of receptor activation.
A better understanding of the mechanisms that lead to ligand bias will improve the design of biasing ligands and help to develop improved drugs. Integration of biological knowledge and experimental data with mathematical models should allow for more mechanistic insights into how signaling network contribute to dynamic, biased signaling. For signal transduction, many properties of recurrent network motifs in signaling have been worked out theoretically and several of those have been verified by experimentals (chapter 5). Regrettably, most of those studies focus on signaling mechanisms associated with receptor tyrosine kinases [268, 294–298]. As more and more (quantitative) data becomes available also in the GPCR field, systems biology will provide a valuable tool to understand the highly dynamic processes of signal transduction initiated by GPCRs.

6.2 Receptors are networks

The results presented in my thesis indicate that the receptor and the downstream signaling network both contribute to biased signaling. The downstream signaling network can amplify, attenuate or even shift biases that arise at the onset of signaling, at the receptor level. In order to exploit and affect the occurrence of ligand bias in clinical applications, both sources of bias need to be investigated.

Network interventions, in order to achieve for instance a change in biased signaling, are particularly hard to predict. Blocking of a signaling route, due to a mutation or the application of a drug, might, for instance, not result in the complete silencing of this signal but can often be bypassed, due to the flexibility within a signaling network as demonstrated by Klinger and coworkers [268].

Network approaches are possible, as we have nowadays a fairly good knowledge of signaling pathways represented in form of network diagrams. We do not have such representations for the allosteric interactions between binding sites on receptors, even though those interactions underlie biased signaling by the receptor. Having such a ‘network perspective’ on the functioning of GPCRs would be highly useful, in addition it maps directly on the conformation-equilibrium model that I presented in this thesis. A unified network-diagram of allosteric ligand-ligand interactions would also allow for a concise summary of experimental knowledge on ligand-ligand interactions and the localization of their binding sites in the 3D structure of the protein. The same diagram building blocks could be used for different receptors to unify the field and facilitate the exchange of information between scientists working on different receptors. A similar endeavor for the depiction of metabolic, signaling, and gene network has proven to be really useful in systems biology [299]. I will shortly introduce my current ideas about such an approach.

Ligand effects on a single receptor can be summarized in a so-called "Black Box" representation in which specifics about the signaling input, i.e. the ligand or combinations of ligands, and the signaling output, i.e. the measured read-out, are
summarized. This is visualized for some CaSR-specific ligands in figure 6.1. From this knowledge, a graphical, more-mechanistic representation of established or predicted allosteric ligand-ligand interactions can be derived and depicted in "Grey Box" diagrams (see figure 6.2 for illustration).

### CaSR-ligand interactions

<table>
<thead>
<tr>
<th>Input</th>
<th>Secondary (<code>modifier</code>)</th>
<th>Ca2+</th>
<th>PHE</th>
<th>Spermine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>output 1: Gq activity</td>
<td>output 2: [Ca2+]i</td>
<td>output 3: ERK phosphorylation</td>
</tr>
<tr>
<td>Ca2+</td>
<td></td>
<td>positive ([Ca2+]i)</td>
<td>positive (EC50 ~ 3.8 mM)</td>
<td>[14]</td>
</tr>
<tr>
<td>PHE</td>
<td>neutral; EC50 ~ 3.3 mM</td>
<td>positive; transient oscillations with frequency ~1 per min</td>
<td>[10]</td>
<td>left-shift of dose-response curve in presence of L-PHE</td>
</tr>
<tr>
<td>Spermine</td>
<td>positive; EC50 ~ 4.0 mM</td>
<td>positive, spermine increases sensitivity to Ca2+ (indirect via IP3 accumulation)</td>
<td>[14]</td>
<td>positive, spermine induces increases in intracell. Ca2+ in dependency of Ca2+o</td>
</tr>
</tbody>
</table>

### Number Reference

1. S. Roth, J. van Unen, J. Goedhart, T.W.J. Gadella, F.J. Bruggeman, manuscript in preparation

**Figure 6.1:** Examples of ligand combinations and their resulting signaling outputs exemplarily for the CaSR. All knowledge about ligand combinations and their signaling effects for a specific receptor can be represented in so-called "black boxes". The primary ligands are listed in the top row, describing orthosteric ligands or ligands that were in the focus of a particular study, e.g. (Ca2+)o, or spermine in my titration experiments. Secondary ligands, that describe modifiers which were added in addition to the main, primary stimulus, are listed in the left column. In this black box, the effects of ligand-ligand interactions are summarized for three CaSR-signaling read-outs, Gq activation, (Ca2+)o, mobilization and ERK phosphorylation. The corresponding references are indicated in the table as numbers in squared brackets and listed below the table. The table can be extended for both primary and secondary ligands, as well as signaling outputs.

Researchers from different fields have a different focus on the same receptor. While a crystallographer sees the receptor from a structural perspective with focus on the position and orientation of amino acids, molecular biologists or physiologists are rather interested in the signaling of an active receptor and its outcome, and all possible nuances in between. In the "Grey Box" approach, we distinguish between a (extracellular) signal input level, which can also be shared between different domains of the receptor (e.g. the Venus Flytrap and the transmembrane domain of glutamate-like GPCRs) and a signal transduction level, at which the intracellular binding proteins are activated by the receptor. Binding sites on both domains can have influence on the information transduction of the receptor, usually measured with experimental read-outs at the network/ signal output level (grey frame). From experiments it is known that the combination of different binding partners influences their effect...
on the signaling capacity of the receptor, represented with arrowheads (activating, positive influence) and bar headed arrows (inhibiting, negative effects). This general, mechanistic representation of a GPCR can act as a summary of the known effects of different ligands on the receptor and its underlying signaling network. Therefore, it can be easily understood and exchanged between scientists of different fields.

In this way, a comprehensive overview of how (allosteric) ligands interact on a GPCR and influence the signaling outcome can be established. I think that this method is a flexible and informative method that can be easily adjusted for different types of receptors, and simplifies the exchange of knowledge between different receptor fields.

![Diagram of receptor function and signaling](image)

**Figure 6.2: Schematic overview of the ‘grey box’-approach.** In this approach, three functional levels of receptor and receptor signaling are distinguished: 1) The (extracellular) signal input level, possibly shared between different domains of the receptor and depicted in blue. 2) The signal transduction level, depicted in green. This is the interface between receptor and the signal transduction molecules. 3) In gray box, the network / signal output level. Usually, experimental read-outs are obtained at this level. This mechanistic representation of a GPCR summarizes the known effects of different ligands and facilitates the exchange of knowledge between scientists of different fields. Crystal structure: human metabotropic glutamate receptor, adapted from [10].

### 6.3 The influence of the cellular context and post-translational modifications on biased signaling

We tend to think of information flow as directing from the outside to the inside of the cell, as depicted in figure 1.1. In this view, it is indeed logical to decompose biased...
signaling into two independent components, the receptor and network contribution, as they occur in sequence. However, a reverse flow has to be appreciated as well as the network can affect the signaling characteristics of the receptor. Accordingly, the receptor should be considered as a conduit [79] as intracellular molecules or processes can alter receptor conformations and modify receptor-level biased signaling. Such a ‘biasing from inside’ has been shown for synthetic molecules acting on the intracellular loops of GPCRs [86]. Further, the binding of intracellular adaptor molecules, besides heterotrimeric G proteins and β-arrestins, might ‘prime’ the receptor for certain extracellular agonists, by altering the ligand binding affinities. As a consequence, the ligand-specificity and biasing-capacity of a receptor becomes cell-context dependent. The same receptor can therefore induce different responses to the same signals in different cells.

An extended version of the conformation-equilibrium model that I presented in chapter 2 indeed indicates that biasing from the inside is a realistic possibility (unpublished results)*. This we were able to show by incorporating the intracellular binding of modulators to active receptor states. We included those binding effects in the partition function, which ultimately determines all the distinct receptor fractions, as presented for the monomeric partition function in equation 6.1.

\[
z_{ij} = 1 + \frac{S}{K_{1,ij}} + \frac{S}{K_{2,ij}} + \frac{S^2}{\alpha_{ij}K_{1,ij}K_{2,ij}} + \frac{A}{K_A} \frac{S}{\alpha_{ij}K_{1,ij}} + \frac{A}{K_A} \frac{S}{\alpha_{ij}K_{2,ij}} + \frac{A}{K_A} \frac{S^2}{\alpha_{ij}K_{1,ij}K_{2,ij}} \tag{6.1}
\]

with \(S\) being the ligand concentration, \(A\) being the adaptor concentration, \(K_A, K_{1,ij}\) and \(K_{2,ij}\) are the adaptor’s and binding sites’ dissociation constants, \(i = \{r, t\}\), and \(j = \{1, 2\}\). \(\alpha_{ij}\) and \(\alpha_A\) represent the effect of allosteric interaction between the ligand binding sites and the adaptor, respectively. The investigation of this extended model has shown that adaptors can (de-)sensitize the receptor for external agonists dramatically, and that the relative abundance of adaptor proteins biases the signaling efficacy of a receptor.

The implication of the receptor as a conduit could explain the cell- and tissue-specificity observed for GPCRs. The multifaceted signaling and expression as well as the diverse roles in physiology and disease of the CaSR could therefore be due to tissue specific intracellular binding partners, in addition to G proteins and β-arrestins. Regulatory proteins of the 14-3-3 family have been shown to interact with the CaSR and modifying its signaling behavior [300] indicating the role of CaSR-binding proteins. Nevertheless, the general role of adaptors on the receptors’ conformation and their impact on cell-type specificity needs to be further investigated.

*M. Slagter, Bidirectional information transfer through transduction mechanisms perceived unidirectional: a paradigm shift. This work has been carried out as a master thesis in 2013 under the supervision of F.J. Bruggeman and S. Roth.
As briefly discussed earlier, phosphorylations are common post-translational modifications of GPCRs that have major impact on signaling [4]. The CaSR, for example, contains multiple serine and threonine residues that are phosphorylated by PKC and other GRKs. Mathematical models indicate that multisite phosphorylations of signaling proteins can promote ultrasensitive responses to inputs [175]. Thus, in principle, double phosphorylations of GPCRs can cause all-or-nothing responses, rather than gradual responses, of receptors, causing them to function as true ON/OFF switches. These effects have also been shown by an extension of the biophysical model of GPCR-conformation equilibria (unpublished results).

All of this suggests that GPCRs should be perceived as sophisticated regulatory proteins capable of bidirectional signaling by integrating intra- and extracellular information and as dynamic bifurcating inputs of cellular signaling. We certainly need more combined theoretical and experimental investigations to elucidate the full signaling capacities of GPCRs.

### 6.4 Conclusions and future perspectives

In summary, this thesis has demonstrated how advanced experimental tools and theoretical considerations help to elucidate the onset and propagation of ligand biased signaling. These investigations are a first step towards a deeper understanding of the roles of receptors and signaling networks in biased signal transduction. I expect that real-time fluorescence-based microscopy techniques, such as FRET, will have a major impact on GPCR research in general and in ligand bias research in particular.

I investigated the biasing effect of different CaSR agonists on only one family of G

\[ \alpha \]

proteins. To gain further insights into the onset of bias at the receptor-G protein level, the – ideally simultaneous – measurements of different G proteins in response to receptor stimulation are required. Tools to study this could comprise sensors consisting of different FRET-pairs in order to observe the activity of two (or more) G proteins simultaneously and to quantify biased signaling at its onset. Moreover, this concept may lead to a deeper understanding of specific biasing properties of receptor-ligand combinations and might result in the identification of general mechanisms leading to bias.

The roles of receptor and networks can be further disentangled by the concurrent use of optical sensors for different signaling processes. Our results on simultaneous measurement of G

\[ \alpha \]

\[ \text{q} \]

and \((\text{Ca}^{2+})\) demonstrate that this kind of experiments are in principle possible. Nevertheless, this approach requires careful testing of crosstalk and other influences of the fluorescent probes on each other. Further, heterologous expression levels of the sensors might be critical and require careful regulation. Stable cell lines expressing the sensors in well-defined ratios would be a next step in these applications.
Real-time microscopy provides detailed information about the dynamics of distant players in the signal transduction cascade. The additional consideration of spatial information on distinct signaling processes would provide further insight on the importance of spatiotemporal component of the onset and propagation of bias. I would propose to investigate signaling in single cells and focus on cell-type-specific signaling properties of GPCRs, possibly with a focus on binding proteins and phosphorylation to characterize cell-context dependent signaling. Analysis of the influence of receptor phosphorylations on signaling could reveal their impact on signaling bias, and verify whether switch-like responses are indeed properties of GPCRs as predicted by models. Systems biology approaches should be applied in order to identify and understand basic mechanisms of biased signal transduction. The combination of experimental findings and theory, as done already for receptor tyrosine kinase initiated signaling, may lead to a deeper, network-level understanding of signaling processes and enable the targeted design and application of biasing drugs.
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