MINIREVIEW—EXPLORING THE BIOLOGY OF GPCRs: FROM IN VITRO TO IN VIVO

G Protein–Coupled Receptor Signaling Networks from a Systems Perspective

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ABSTRACT

The signal-transduction network of a mammalian cell integrates internal and external cues to initiate adaptive responses. Among the cell-surface receptors are the G protein–coupled receptors (GPCRs), which 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 underappreciated receptors in systems-biology research.

Introduction

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 toward this goal (Oda et al., 2005; Hyduke and Palsson, 2010)], 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 (Aldridge et al., 2006; Kholodenko, 2006; Du and Elemento, 2015).

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 (Dehmelt and Bastiaens, 2010; Kholodenko et al., 2010). 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

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ABBREVIATIONS: GAP, GTPase-activating protein; GEF, guanine exchange factor; GPCR, G protein–coupled receptor; GRK, G protein receptor kinase; MAPK, mitogen-activated protein kinase.
Why G Protein–Coupled Receptors 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 coexist in a thermodynamic equilibrium, which shifts under the influence of signal binding (Venkatakrishnan et al., 2013). Agonist binding will induce a conformational change of the receptor that activates intracellular signaling, whereas 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. It was long 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 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.

Classic approaches for equilibrium-binding models of cooperative proteins immediately apply to GPCRs (De Lean et al., 1980; Iyengar et al., 1980; Black and Leff, 1983; Hall, 2000; Changeux, 2013; Roth and Bruggeman, 2014) and can account for signal integration by receptors—so called “combinatorial ligand-bias.” This is in contrast to, for instance, receptor tyrosine kinases that require phosphorylations, a kinetic nonequilibrium 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 (Goldbeter and Koshland, 1981; Koshland et al., 1982; Blüthgen et al., 2006). Likewise, biochemical equilibrium-models of competitive binding describe the competition of immediate downstream proteins, such as G proteins, 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 focused much more on receptor tyrosine kinases–induced cellular signaling (the RAF-MEK-ERK axis) than on GPCR-activated networks. 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 (Fig. 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 tables, in which we explain a finding in quantitative systems biologic 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.

Quantification of the Membrane Processes

In systems biology, emphasis is put on quantitative studies (Aldridge et al., 2006). In the 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 (Heitzler et al., 2012). 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, although their abundances (expression level) can greatly vary.

In addition, monomer mobility and oligomerization affinities of GPCRs are key factors, because they determine the active fraction of receptors.

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 (Lohse et al., 2014). Receptors are expressed at several hundred to several thousands of copies per cell. They are surprisingly mobile with often localization-independent diffusivities (Hern et al., 2010; Lohse et al., 2012). The oligomerization kinetics for several members of distinct GPCR families has been determined as well (Fig. 2) (Hern et al., 2010; Kasai et al., 2011; Kasai and Kusumi, 2014). In Table 1, we discuss the quantitative aspects of measured dimerization kinetics. We emphasize that at this stage it is not at all clear whether dimeric receptor complexes are always the active species.

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 (Changeux, 2013). 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 stabilizes 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

TABLE 1

Membrane diffusion and the time scales of complex-formation equilibria of receptors

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
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<tbody>
<tr>
<td>k⁺</td>
<td>2 × 10⁻³ copies⁻¹ 𝜇⁻¹</td>
</tr>
<tr>
<td>K₀</td>
<td>1.0138 copies⁻¹ cell⁻¹</td>
</tr>
</tbody>
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indicating that the dimer, denoted by m₂, lifetime is about 0.1 seconds (1/k⁻); 10 dimers fall apart every second. The total number of receptor monomers per cell, m₁, was 6000. At thermodynamic equilibrium, when K₀ = m³/m₂, and given m₂ = m + 2m₁, the equilibrium concentrations of the monomer and the dimer become m = 3534 copies/cell and m₂ = 1233 copies/cell: less than 50% of the monomers exist as dimers. The lifetime of a monomer equals 0.4 seconds (k⁺m₂/m₁). The diffusion coefficient of a monomer in the membrane sets the upper limit of the association rate constant (Sheng and Sablo, 1982) to 3 × 10⁻⁶ cm²/s (Lauffenburger and Linderman, 1993). The k⁺ of FPR is very close to this limit. Given a realistic diffusion coefficient (D) of 0.1 μm²/s for a GPCR (Hern et al., 2010), the average distance that a GPCR travels in a membrane in one second equals √4Dt = 0.6 μm.
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 \( \tau_1 \) (\( \tau_7 \) denotes the total receptor concentration) (Roth and Bruggeman, 2014):  
\[
\tau_{1,s} = \frac{(s^2 \alpha K_R^2)}{(s^2 \alpha K_R^2 + L(s_1^2 + \ell^2) + L(s_2^2 + \ell^2))},
\]

for \( s \), the conformation-dependent affinity of the subunits for the ligand \( K_{R,1} \), the allosteric factor \( \alpha \), the intracellular-domain conformation equilibrium constants \( s_1 \) and \( s_2 \) and, finally, on the receptor equilibrium constant \( L \). The power \( ^{\alpha} \) 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 \( \tau_7 \) raised to \( \alpha \) (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,  
\[
\tau_{1,s} = \frac{(s^2 \alpha K_R^2)}{(1 + s^2 \alpha K_R^2)}.
\]

This equation gives a sigmoidal dependency of the active receptor fraction on the ligand concentration. The Hill coefficient of this equation equals \( \frac{\partial \ln \tau_{1,s}}{\partial \ln s} \) at \( s = s_{0,5} \) when \( r_{1,s} = r_{7,T}/2 \). This model displays all the features associated with ligand bias (Fig. 3) (Roth and Bruggeman, 2014). 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.

![Fig. 3](https://example.com/fig3.png)  
*Fig. 3. Illustration of ligand bias and ultrasensitivity. (A) 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 cause a deviation from this linear relation and are represented by the curved, dashed lines. (B) Ultrasensitivity is represented as a sigmoidal dose-response curve (solid line), whereas a hyperbolic curve, characteristic for a hyperbolic "Michaelis-Menten" response is not ultrasensitive (dashed line)."
In addition to classic, extracellular signals, peptides, peptidcs, and nanobodies that bind the receptor intracellularly can also affect the coupling GPCR to signaling molecules (Quoyer et al., 2013; Staus et al., 2014; reviewed in Shukla, 2014). 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 (Kinzer-Ursem and Linderman, 2007), 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 (Kinzer-Ursem and Linderman, 2007). 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 (Falkenburger et al., 2010). The GPCR field has advanced to enzymatic activity presumably resides on the GPCR (Oldham and Hamm, 2008). The activating conformation change of the GPCR causes it to have guanine exchange factor (GEF) activity. Upon activation, the G protein can dissociate from the complex and activate downstream signaling until it is inactivated by its GTPase activity, which is 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 balance between GEF and GAP activities (Oldham and Hamm, 2008). In Table 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 ultrasensitivity with respect to signals (Fig. 3) (Goldbeter and Koshland, 1981; Blüthgen and Hamm, 2008). Similarly, a model of the M1 muscarinic receptor activation/inactivation cycle can in principle show the same behavior—although it is not strictly a covalent-modification cycle—with respect to signals acting on the GPCR.

In case of ultrasensitivity, 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 coefficient exceeding 4). Switch-like, zero-order, ultrasensitive behavior occurs when both the GEF and the GAP enzymes are saturated with their substrates—$[G_{GDP}] > K_{Mena}$ and $[G_{GTP}] > K_{GAP}$—such that they operate in their zero-order regime ($K_{M}$ denotes the Michaelis-Menten constant of the enzyme) (Table 3). Trunnell et al. (2011) present an experimental illustration of ultrasensitivity.

Turotto et al. (2008) developed a kinetic model of a G protein covalent-modification cycle and found ultrasensitivity in the calculated dose-response curves. Ultrasensitive responses have

### 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 (Oldham and Hamm, 2008). The activating conformation change of the GPCR causes it to have guanine exchange factor (GEF) activity. Upon activation, the G protein can dissociate from the complex and activate downstream signaling until it is inactivated by its GTPase activity, which is 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 balance between GEF and GAP activities (Oldham and Hamm, 2008). In Table 3 we show some of the basic aspects of such processes.

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### Table 3

<table>
<thead>
<tr>
<th>G protein activation and sensitivity of its GTP-bound state ($[G_{GTP}]$) to GPCR ligands</th>
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<tr>
<td>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,</td>
</tr>
<tr>
<td>$R^4 + [G_{GDP}] \rightarrow R^4 + [G_{GTP}]$ (3)</td>
</tr>
<tr>
<td>$GAP + [G_{GTP}] \rightarrow [GAP_{GTP}] \rightarrow [GAP] + [G_{GDP}]$ (4)</td>
</tr>
<tr>
<td>Here $R^4$ 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:</td>
</tr>
<tr>
<td>$v_{GEF}(G_{GDP}, G_{GTP}) = k_{GEF} R^4 \frac{[G_{GDP}]}{1 + [G_{GDP}] + [G_{GTP}]}$ (5)</td>
</tr>
<tr>
<td>$v_{GAP}(G_{GDP}, G_{GTP}) = k_{GAP} [GAP] \frac{[G_{GDP}]}{1 + [G_{GDP}] + [G_{GTP}]}$ (6)</td>
</tr>
<tr>
<td>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 ligand that binds to the receptor, is $\frac{\delta \ln [G_{GTP}]}{\delta \ln R^4} = \frac{1}{\delta \ln [G_{GDP}]} \left( \frac{1}{[G_{GDP}]} \left( \frac{1}{k_{GAP}} + \frac{1}{k_{GEF}} \right) \right)$ (7)</td>
</tr>
<tr>
<td>The $\epsilon$ coefficients are generalized kinetic orders [or elasticity coefficients (Kholodenko et al., 1997)] of the reactions: $\epsilon_{GEF} = \frac{\delta \ln v_{GEF}}{\delta \ln [G_{GDP}]}$. When they are close to zero, the enzyme operates in its zero-order regime ($\epsilon_{GEF} = 0$) and zero-order ultrasensitivity occurs (Blüthgen et al., 2006). This is most noticeable when we assume both enzyme rates insensitive to their product concentrations; then $\frac{\delta \ln [G_{GTP}]}{\delta \ln R^4} = r_{GEF}^{\text{f}} = \frac{1}{\epsilon_{GEF} + \epsilon_{GAP}}$, 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, $R_S^{\text{f}}$, can be decomposed into $R_S^{\text{f}} = \frac{\delta \ln [G_{GTP}]}{\delta \ln R^4} = \frac{\delta \ln [G_{GTP}]}{\delta \ln [G_{GDP}]}$, can be decomposed into (Kholodenko et al., 1997):</td>
</tr>
<tr>
<td>$\frac{\delta R^4}{\delta \ln R^4} = \frac{[G_{GTP}]}{[G_{GDP}]}$ (8)</td>
</tr>
<tr>
<td>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, $r_{GEF}^{\text{f}}$, and the G protein sensitivity, $r_{GAP}^{\text{f}}$. Therefore, &quot;sensitivity amplification&quot; (Goldbeter and Koshland, 1981; Koshland et al., 1982; Kholodenko et al., 1997) occurs when both $r$ values exceed 1.</td>
</tr>
</tbody>
</table>
found experimentally with PIP3 activation (Karunarathne et al., 2013). In a combined experimental and modeling study, Karunarathne et al. (2013) demonstrated that PIP3 concentrations are ultram sensitive with respect to GPCR activation.

The phosphorylation of GPCRs by GRKs and the associated dephosphorylation events can also lead to G protein sensitivities. GRKs have a broad influence on GPCR signaling (Jiménez-Sainz et al., 2006). Systems biology studies on GRK-regulated GPCR activity (Vayttaden et al., 2010; Heitzler et al., 2006) indicate the versatility GRK regulation and are examples of the insightful combinations of experiments and modeling.

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 are initiated at the cell surface are transduced via cascades of covalent modification cycles, among 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 (Morrison, 2012). GPCRs activate the ERK, c-Jun N-terminal kinase, and p38 MAPK cascades using spatially and temporally distinct pathways that are dependent on either G proteins or β-arrestins (DeWire et al., 2007). Direct MAPK activation via Ga is transient, β-arrestin independent, and can involve PKC activity (Goldsmith and Dhanasekaran, 2007). The other pathway leading to MAPK activation is triggered by the recruitment of β-arrestins as scaffolding molecules (Lefkowitz and Shenoy, 2005; Goldsmith and Dhanasekaran, 2007). For β-arrestin-mediated ERK signaling, β-arrestins scaffold all three MAPK cascade kinases, Raf, MEK, and ERK (DeFea et al., 2000). Because β-arrestins interact with components of the clathrin-mediated endocytic pathway, GPCRs bound to β-arrestins are targeted to clathrin-coated pits. For different GPCRs, β-arrestins either rapidly dissociate and receptors recycle to the plasma membrane, or GPCR-β-arrestin complexes stay together on the surface of endocytic vesicles. In either case, β-arrestin 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 (Kholodenko, 2002). 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 (Ahn et al., 2004).

### Table 4
Sensitivity of networks to signals: cascades, feedback, and feedforward networks

<table>
<thead>
<tr>
<th>Signal</th>
<th>Cascades</th>
<th>Feedback</th>
<th>Feedforward</th>
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<td>Sensitivity to signals</td>
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</tr>
<tr>
<td>Sensitivity of networks</td>
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<td>Feedback</td>
<td>Feedforward</td>
</tr>
</tbody>
</table>

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 (Khodolenko et al., 1997; Bruggeman et al., 2002), with A-to-D as concentrations of signaling proteins,

\[ \frac{dA}{dt} = \frac{r_D}{r_C} - \frac{r_D}{D} \]

where \( r_D \) and \( r_C \) are the rate constants for the production of D and C, respectively. Sensitivity profiles are also remarkably different. In human embryonic kidney-293 cells, G protein–dependent ERK1/2 activation is transient, rapidly reaching a peak at about 2 minutes and descending to low levels after about 10 minutes after stimulation, whereas activation via β-arrestins is sustained until at least 90 minutes (Ahn et al., 2004). 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.

The same principle of β-arrestin scaffolding leads to GPCR-induced NFκB signaling (Sun and Lin, 2008), a signaling cascade that is also controlled by a series of posttranslational modifications. The MAPK system has been studied extensively and several interesting signaling features have been found, such as oscillations, drug robustness, and ultrasensitivity (Ferrell and Machleder, 1998; Shankaran and Wiley, 2010; Sturm et al., 2010a; Fritsche-Guenther et al., 2011).

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 (Goldbeter and Koshland, 1981; Huang and Ferrell, 1996; Kholodenko et al., 1997; Ferrell and Machleder, 1998). For instance, the MAPK pathway of *Xenopus* oocytes was found to have a Hill coefficient of 8, which is truly a switch-like response (Ferrell and Machleder, 1998), and believed to be due to "sensitivity amplification." Sensitivity amplification can be understood in terms of intuitive theory (Kholodenko et al., 1997), an example of this is shown in Table 4, and with mathematical models based on enzyme kinetics (Huang and Ferrell, 1996). How heightened sensitivity arises in cascades follows from basic principles of the enzyme kinetics of the associated kinases and phosphatases (Blüthgen et al., 2006; Ferrell and Ha, 2014a). The cascade sensitivity equals the product of the sensitivities of the individual covalent-modification cycli in the cascade (Fig. 4; Table 4) (Kholodenko et al., 1997; Bruggeman et al., 2002). The sensitivity of a single covalent modification cyclus depends on the extent of the saturation of the kinases and phosphatases (Table 3).

Activation of signaling proteins by double phosphorylation, such as of MEK and ERK leading to MEKP2 and ERKP2, can cause to heightened sensitivity at the level of a single cascade element (Markevich et al., 2004). 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 ERKP2, with respect to the input, e.g., MEKP2, with hysteresis properties. Bistability is explained in Fig. 7 and has been found in mathematical models for a number of signaling networks (Bhalia and Iyengar, 2001; Markevich et al., 2004; Qiao et al., 2007; Ravichandran et al., 2013). 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 (Laslo et al., 2006; Ferrell, 2012).

Ultrasensitivity, sensitivity amplification, and bistability (Ferrell and Ha, 2014a,b,c) 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 (Aldridge et al., 2006).

**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 (Volinsky and Kholodenko, 2013). 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 (Blüthgen et al., 2007). The study of negative feedbacks has revealed their impact on signaling and appear to contribute to adaptation, robustness, and oscillations (Kholodenko, 2006) (Fig. 5). Oscillatory dynamics has been found in a range of signaling networks, e.g., during NFκB, ERK, and intracellular-Ca²⁺ signaling (Hoffmann et al., 2002; Nelson et al., 2004; Shankaran et al., 2009; Dupont et al., 2011), 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 (Pitcher et al., 1999) or via other mechanisms (Ferguson, 2007). An important example of negative feedback regulation of GPCR-activated signaling is found in cAMP signaling, where downstream activation activates phosphodiesterases that reduce cAMP levels (Sassone-Corsi, 2012).

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 Ga (Fig. 6) (Kummer et al., 2000) and the internalization rate of β subunits (Giri et al., 2014). Various mathematical models exist that focus on different aspects of calcium oscillations (reviewed in Dupont et al., 2011). Cross-talk between Ga and Ga, regulated signaling, investigated with a modeling approach by Siso-Nadal et al. (2009), 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 fluorescence resonance energy transfer sensors should give more insight into oscillations of G protein activation. It was also found that green fluorescent protein–tagged ERK displayed oscillations between cytosol and nucleus over a broad range of ligand concentrations (Shankaran et al., 2009). Through modeling and experiments it was concluded that the negative feedback from ERK to upstream proteins underlies these oscillations, which are likely functional during development (Shankaran et al., 2009; Shankaran and Wiley, 2010). In NFκB signaling, a strong negative feedback of IkBα is leading to oscillations in NFκB (Hoffmann et al., 2002). Thus, the function of NFκB as a transcription factor might therefore depend on the characteristics of its oscillations, leading to dynamic control of gene expression (Nelson et al., 2004). Direct involvement of negative feedback of GPCRs in signaling oscillations has, as far as we know, not yet been established.

Other than 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 used in engineering to design robust systems (Sturm et al., 2010b). 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 Fig. 5) 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 (Fritsche-Guenther et al., 2011). It also contributed to robustness to a MEK inhibitor during medical treatment (Sturm et al., 2010b; Fritsche-Guenther et al., 2011). For colorectal cancer cells, it was shown that the negative feedback in EGFR signaling led to cross-talk 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 (Sturm et al., 2010b; Klinger et al., 2013). 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,

![Fig. 6. Oscillatory signaling dynamics can be induced by a negative feedback (Kummer et al., 2000). (A) Oscillations of the concentrations of signaling molecules. (B) Detailed view of the cytosolic calcium oscillations.](molpharm.aspetjournals.org)
studies indicate that the absence of a feedback, e.g., by a mutation in Raf, can sensitize cells to inhibitors (Sturm et al., 2010b; Fritsche-Guenther et al., 2011) (Table 5). Mathematical models and network concepts are therefore providing new ways for studying signaling networks and predicting drug responses.

**Positive Feedback in Signal Transduction Cascades: From Gradual to All-or-One Bistable Responses**

Although 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 (Kholodenko, 2006; Ferrell and Ha, 2014a,b,c). 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) (Ferrell and Ha, 2014a,c), and generation of cellular subpopulations (phenotypic diversification, cellular differentiation) (Ferrell, 2012; Ferrell and Ha, 2014c) (see Fig. 7). 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 (Ferrell and Machleder, 1998; Xiong and Ferrell, 2003) during immune responses (Mariani et al., 2010), cellular differentiation (Chickarmane et al., 2006; Glauche et al., 2010), and the MAPK pathway (Ferrell and Machleder, 1998; Bhalla et al., 2002). A requirement for bistable behavior is that the signaling cascade displays sensitivity amplification along the feedback loop (Table 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

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**TABLE 6**

Fold change detection by a molecular activation cascade

A simple model of a feedforward motif illustrates the principle of fold change detection (Goentoro et al., 2009). We consider a system with the signal $S$ and two signaling proteins $X$ and $Y$, their concentrations change according to:

$$
\frac{dx}{dt} = k_1 x - k_2 x,
$$

$$
\frac{dy}{dt} = k_3 y - k_4 y.
$$

$S$ 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:

$$
x_s = \frac{k_1 s}{k_2},
$$

$$
y_s = \frac{k_3 s}{k_4}.
$$

Note that a change in $s$ does not affect $y_s$, $y_s$ 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 normalize the concentration with respect to their basal, steady-state levels at reference concentration $s$,

$$
F_x = \frac{s}{x_s}, F_y = \frac{y_s}{y_s} = 1.
$$

with $s$ 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:

$$
\frac{d}{dt} F_x = F_x - F_s,
$$

$$
\frac{d}{dt} F_y = F_y - F_s,
$$

with $r = k_2$. 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 gives 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, because they do not affect signaling outcome as long as the outcome is considered normalized to the basal concentrations.
in the absence of feedback loops or cascade interactions (Markevich et al., 2004). Legewie et al. (2007) reported that complex formation in signaling cascades, for instance via scaffolds, can cause bistability. 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.

**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. Because cells do not generally 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 NFkB, intracellular Ca2+, and ERK oscillations and of bistability during signaling and cellular differentiation relied on fluorescent reporters (Hoffmann et al., 2002; Nelson et al., 2004; Sigal et al., 2006; Cohen-Saidon et al., 2009; Shankaran et al., 2009).

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 (Sigal et al., 2006; Raj and van Oudenaarden, 2008; Cohen-Saidon et al., 2009; Spencer et al., 2009), which can be understood from basic biophysics (Schwabe and Bruggeman, 2014). This phenomenon has been termed “noise” (Raj and van Oudenaarden, 2008). Feedbacks in networks can both reduce and enhance noise. A consequence of noise is that cells can vary in their individual responses to physiologic triggers (Yuan et al., 2011), including drugs (Spencer et al., 2009). Kempe et al. (2015) 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 (Balázsi et al., 2011).

**Fold-Change Detection by Networks Compensates for Stochastic Fluctuations in Protein Levels**

Because of inevitable fluctuations in molecular activities (Elf and Ehrenberg, 2003; Paulsson, 2004), cells exploit regulatory circuitry to reduce the harmful impacts of noise, occurring in signal transduction and transcription (Perkins and Swain, 2009; Cheong et al., 2011). In this way, cells increase the reliability of information transfer in signaling networks (Levchenko and Nemenman, 2014). Negative feedback is an example of a noise-reduction mechanism (Bruggeman et al., 2009).

Another mechanism for noise reduction is fold-change detection (Table 6). Networks that detect fold changes have the same dynamics upon the same fold change in the stimulus, regardless of its basal level (Goentoro et al., 2009); this is visualized in Fig. 8. An (incoherent) feedforward loop in a signaling network gives rise to fold-change detection (Goentoro et al., 2009), as well as other network motifs (Shoval et al., 2010). Fold-change detection has been reported for mammalian signaling (Cohen-Saidon et al., 2009; Goentoro et al., 2009). For instance, Cohen-Saidon et al. (2009) studied the dynamics of nuclear ERK2 upon EGF stimulation using a genetically encoded, yellow fluorescent protein–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 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-feedback loop network (Cohen-Saidon et al., 2009; Goentoro et al., 2009).

Hart et al. (2013) 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 (Table 2), and it is therefore tempting to speculate that some GPCRs may have fold-change–detecting properties as well.

These studies indicate that the operation of some signaling systems should not be appreciated 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.

**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. Combina-
tion of new techniques, such as G protein fluorescence reso-

nance energy transfer 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 in-

dicating 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 pro-
teins, signaling cascades, and interwoven feedback and

feedforward circuitry lead to counterintuitive, functional

signaling dynamics that are best understood with mathemat-

ical models and single-cell experiments. We therefore expect that the next developments in the GPCR field will move toward 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 Klinger et al., 2013).

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 cellular outputs. For example, we illustrated how ultrasensitivity arises and can result from cascade sensitivity amplification. An important aspect was how feedbacks shape signaling functions in a cell, including oscillations and robustness. We emphasize that many of these network-centered studies do not yet focus on GPCRs, although 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 cross-talk between membrane receptors and their induced signaling pathways (Lowes et al., 2002), 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.

**Authorship Contributions**

Wrote or contributed to the writing of the manuscript: Roth, Khodolenko, Smit, Bruggerman.

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