Impact of upstream and downstream constraints on a signaling module’s ultrasensitivity

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Abstract

Much work has been done on the study of the biochemical mechanisms that result in ultrasensitive behavior of simple biochemical modules. However, in a living cell, such modules are embedded in a bigger network that constrains the range of inputs that the module will receive as well as the range of the module’s outputs that network will be able to detect. Here, we studied how the effective ultrasensitivity of a modular system is affected by these restrictions. We use a simple setup to explore to what extent the dynamic range spanned by upstream and downstream components of an ultrasensitive module impact on the effective sensitivity of the system. Interestingly, we found for some ultrasensitive motifs that dynamic range limitations imposed by downstream components can produce effective sensitivities much larger than that of the original module when considered in isolation.

Keywords: signaling, ultrasensitivity, dynamic range, transfer function

1. Background

Cells continuously sense external and internal cues using specific molecular components in order to elaborate appropriate responses which help to cope with a challenging environment. During the last 15 years, a great deal of experimental and theoretical effort has been devoted to understand the underlying biochemical reaction network responsible for these capabilities. Much of the advance in the field, along with the development of new areas such as synthetic biology, was conceived under a system-level perspective in which signal sensing, transduction, and information processing mechanisms are analyzed in terms of interacting modules or minimal functional motifs (Tyson et al 2003, Benner and Sismour 2005, Alon 2006). The modular description of molecular biochemical networks is based on the hypothesis that system-level properties may in fact be derived from the analysis of interactions among operationally defined subsets of molecular species, i.e. functional modules (Hartwell et al 2000, Koloddenko et al 2002, Sneppen et al 2010).

Among the plethora of biologically plausible functional units sigmoidal input-output response modules constitute one of the fundamental motifs found in several cellular contexts (Zhang et al 2013). Sigmoidal responses have a threshold input level below which low output signals are produced, and above which high response levels are achieved. The steepness of the transition between low to nearly maximum response is referred to as the module’s sensitivity. Biochemical modules with steeper transitions than that obtained in a hyperbolic Michaelis-Menten
response are called ultrasonic (Goldbeter and Koshland 1981), and in the extreme, they behave like an all or none (digital) switch. At a first approximation, ultrasonic responses may be described by a Hill equation of the form: $y = A x^m/(EC50^m + x^m)$, where $x$ and $y$ represent the system’s input and output, respectively. The parameter EC50 is associated to the input level for which half-maximal output is achieved, $A$ is the maximal output, while $nH$ is known as the Hill coefficient, and is usually used to quantify the global sensitivity of the corresponding transfer function.

At the molecular level, several mechanisms can produce ultrasonic or even switch-like responses. For example, under a competition by stoichiometric inhibitor scenario (Ferrell 1996, Buchler and Louis 2008) switch-like responses could arise because the input signal has to overcome the effect of the inhibitors in order to produce appreciable responses. The same kind of mechanism could produce ultrasonic behavior when translocation sufficiently raises the local concentration of a signaling protein to partially saturate the enzyme that inactivates it (Ferrell 1998). In covalent cycles, so called zero-order ultrasensitivity could arise when kinases and phosphatases work under saturation and imbalances between phosphorylation and de-phosphorylation rates drive drastic changes in substrate phosphorylation levels (Goldbeter and Koshland 1981). Multistep activation processes—like multisite phosphorylation (Ferrell 1996, Markveich et al 2004, Gunawardena 2005)—or ligand binding to multimeric receptors (Rippe 1997)—may also result in ultrasonic dose-response curves. In these cases, the ultrasensitivity arises as a result of a common regulation of two or more concomitant biochemical processes.

At a system level, sigmoidal modules might be used to implement the switch-like responses that are at the core of cellular decision systems, as well as to provide the necessary nonlinearities upon which more complex behaviors, such as adaptation (Srividhya et al 2011), bistability (Angeli et al 2004, Ferrell and Xiong 2001), and oscillations (Kholodenko 2000) can emerge. Many theoretical concepts and operational tools for the analysis of the sensitivity features of functional modules have been developed for the analysis of signal propagation through molecular cascades. A module-based analysis of this kind of systems relays on Hartwell’s idea of functional modules. They can be conceptualized as discrete entities, composed by different types of molecular species, capable of introducing a well-defined and identifiable functionality into the biochemical system they are embedded on.

Within a modular-based perspective, Goldbeter & Koshland (GK) described in a series of influential contributions sensitivity amplification capabilities of covalent modification cycles, and provided analytical tools to quantify the total amplification in multicyclic cascades (Goldbeter and Koshland 1981, Goldbeter and Koshland 1982). Rooted in GK’s work, a similar framework was presented by (Kholodenko et al 1997) to quantify information transfer in signal transduction pathways. Using concepts borrowed from metabolic control analysis they used response coefficients, which describe fractional changes in response levels due to fractional changes in input signals. They showed that, in a multi-level arrangement of consecutive signal processing units (i.e. in a cascade lineup), the global response coefficient equals the product of the response coefficients of each level of the cascade (Kholodenko et al 1997, Brown et al 1997). Interestingly, J.E. Ferrell pointed out in a complementary contribution that the overall global sensitivity (assessed by its Hill coefficient $n$) of two consecutive Hill-type modules, could be at most the value obtained by the multiplication of individual module sensitivities (i.e.: $n \leq n_1 \cdot n_2$). The actual value depended on the relationship between the first module’s output level and the concentration needed to achieve maximal activation of the second module (Ferrell 1997). As a consequence of this kind of analysis, a classification scheme was coined in terms of the sub-multiplicative, multiplicative or supra-multiplicative character of module-based systems, for cases where the overall system sensitivity, $n$, was found to be lower, equal or larger than the product of the constituent module’s ultrasensitivities, $n_i$ (Racz and Slepchenko 2008, O’Shaughnessy et al 2011).

The very notion of module implies that it should be somehow separable from the rest of the system. This separation depends on chemical isolation, which can originate from spatial localization or from chemical specificity (Hartwell et al 2000). For instance, neglecting enzyme-substrate complex formation, a MAPK cascade can be analyzed in terms of three independent modules, characterized by their respective transfer functions, arranged in a linear setup. Importantly, when enzyme concentrations cannot be disregarded, a mechanistic description that takes into account every possible interaction between cascade molecular components should be considered instead (Ventura et al 2008).

As modular-based analysis, mechanistic approaches also support the idea that the biological value of this ubiquitous motifs relies in their ability to convert graded or modestly ultrasonic responses into markedly switch-like responses. Using a mechanistic model, Ferrell and collaborators could gain system-level insights from their experimental study of steady-state responses of the MAPK cascade in Xenopus oocyte extracts (Huang and Ferrell 1996). With the aid of their biochemical model they were able to quantify the incremental sensitivity amplification along the cascade levels, and concluded that the cascade arrangement might contribute to the all-or-none character of oocyte maturation.

As already stated, some limitations of the module based description are expected to be found for cases where enzyme concentrations could not be disregarded. Bluthgen et al showed that, due to sequestration effects (i.e. accumulation of active kinase in complex with its substrate), the response coefficients in the Ferrell model (Huang and Ferrell 1996) were lower than expected from the modular-based description of the same cascade (Bluthgen et al 2006). In connection with this behavior, De Racz & Slepchenko analyzed multilevel cascades of activation-deactivation cycles, and found that the sensitivity lost due to sequestration does not occur when the covalent modification cycles are asymmetric (in particular when phosphatase enzymes are saturated but not the kinases) (Racz and Slepchenko 2008). Taking into consideration
effects like sequestration of shared components\(^4\) (Ventura 
et al 2008, Del Vecchio et al (2008)), it is clear that the 
integration of a given well-characterized module with 
upstream and downstream components often alters its information-
processing capabilities. This is relevant when trying to 
understand the behavior of a modular processing unit 
immersed in its physiological context, or when engineering functional modules for synthetic biology purposes (Ray 
et al 2011, Kittleson et al 2012, Ang et al 2013).

In the present contribution, we focused on the effect of a 
specific factor that can qualitatively alter the effective performance of sigmoidal modules, i.e. the actual response dynamic ranges between consecutive modules. In connection with this, Slepchenko and collaborators briefly commented on the effect of a so-called ‘misalignment’ between two coupled covalent cycles (Racz and Slepchenko 2008); and along the same lines, Bluthgen et al (Bluthgen and Herzl 2003) also identified these effects in the case of MAP kinase cascades. Here, we present a general framework that fits these previous observations and that enables a systematic analysis of the system level sensitivities for modules with sigmoidal input-output transfer functions. In particular, we will explore the effective sensitivity of a three-module system as a function of a) the range of inputs that a central sigmoidal module receives from an upstream component (upstream or input constraint), and b) the range of this module’s output that a downstream component is able to detect (downstream or readout constraint). As we will show, these constraints on input and readout dynamic ranges can result in effective sensitivities much lower or, quite unexpectedly, larger, than the sensitivity of the original module considered in isolation.

We adopted in this work a deterministic methodology to describe the phenomenology displayed by the analyzed motifs. We acknowledge that the probabilistic character of biochemical reactions could induce non-negligible fluctuations in species concentration in the limit of small number of molecules (Thattai and Van Oudenaarden 2001, Paulsson 2005). Sensitivity attenuation (Berg et al (2000), Voliotis 2014), low-pass filtering capabilities in long cascades (Hooshangi et al 2005), and other interesting noise-related phenomena have already been reported in the literature (Ferrell 1998, Thattai and Van Oudenaarden 2002, Raj and Van Oudenaarden 2008, Ray and Igoshin 2012). Even though protein fluctuations are ubiquitous in biological systems, we found that our deterministic approach—that becomes accurate whenever proteins are present at large numbers (Berg et al (2000))—still provide useful insights on the analyzed system’s behavior.

The paper was organized as followed. We began with a brief introduction to basic concepts and operational tools used to quantify the sensitivity of general sigmoidal transfer functions. Then, we introduced the considered methodological setup and the relevant parameters used in order to quantify input and readout dynamic range restrictions. A detailed analysis of input and output dynamic range constraints on several ultrasensitive sigmoidal motifs is then presented. In the last section, we discussed particular and general observed trends and finally conclusions were drawn.

2. Basics on sigmoidal response curves

A variety of mechanisms can produce ultrasensitive and even switch-like responses. Even though the detailed shape of each stimulus–response curve differs from mechanism to mechanism, all of them are associated to processes in which, in the vicinity of certain threshold value of the input signal, small relative variations in the stimulus can produce large relative changes in the response.

As we already mentioned, ultrasensitive responses may be described by a Hill equation and even in the absence of any formal mechanistic foundation, this function is widely used as a phenomenological mathematical description of ultrasensitive processes. In general, the following operational definition of the Hill coefficient may be used to calculate the sensitivity of sigmoidal modules:

\[
\frac{d y}{d x} = \frac{y^m}{x^m + K^m} 
\]

where \(y\) is the signal value producing the \(r\) fraction level of maximal response. The Hill coefficient measures the fold increase needed to drive the output from the non-activated state (arbitrarily defined as the 10% of the maximum output) to the fully activated state (defined as the 90% of the maximum output). The \(r\) value in equation (1) is set to produce a \(n_H = 1\) for hyperbolic response functions (such as those obtained with simple biochemical reactions that follow the Michaelis-Menten approximation). This means that for this kind of transfer functions, an \(81\)-fold change in input signal is necessary to drive the system output from 10% to 90% of its maximal response. Response functions with \(n_H\) values higher than 1 are called ultrasensitive responses. They produce the same amount of system’s output change, with a much smaller fold change in the input signal (e.g. in a \(n_H = 2\) case, only a 9-fold input signal increase is needed to achieve the aforementioned level of change in the output level).

It is worth noting that, differently from the paradigmatic Hill function case, many signaling motifs display non-symmetrical response functions. As will be further discussed in next sections, these kind of transfer functions could arise in many relevant biochemical contexts such as in covalent modification cycles, multistep activation modules, and when molecular titration or stoichiometric inhibition are dominant processes in a particular bio-molecular motif.

For covalent cycles described by Goldbeter-Koshland equations, imbalance in enzyme saturation levels induces asymmetries in the sensitivity before and after the \(EC_{50}\) value (Gomez-Uribe et al 2007). In this case transfer functions in
which the sensitivity displayed for lower input values is larger than the one observed for high input values, can be observed when the phosphatase is in saturation but not the kinase (see supplementary material SM1 (stacks.iop.org/PB/11/066003/mmedia)) (Gomez-Uribet al. 2007). Motifs involving molecular titration mechanisms are also relevant examples of this kind of non-symmetrical response functions. In these units ultrasensitivity could arise when the inhibitor exists in a large quantity and the signaling molecule has a higher binding affinity for the inhibitor than for the target molecule (Buchler and Louis 2008; Zhang et al. 2013). A small increase in signaling molecule concentration cause an explosive response when free inhibitor molecules, forming inactive complexes, are exhausted. This produces a much larger sensitivity for input values below the EC50 input level, than for large input signals, for which a smoother saturation behavior is observed.

Here we proposed to extend the sensitivity quantification provided by equation (1), in order to provide a better characterization of these kind of non-symmetrical response functions. In this way, left, \( n_{H}^{L} \), and right, \( n_{H}^{R} \), Hill coefficients can be defined as:

\[
F(x) = \begin{cases} 
A \frac{x^{n_{H}^{L}}}{EC50^{n_{H}^{L}} + x^{n_{H}^{L}}} & \text{if } x \leq EC50 \\
A' \frac{x^{n_{H}^{R}}}{EC50^{n_{H}^{R}} + x^{n_{H}^{R}}} & \text{if } x > EC50
\end{cases}
\]

(2)

Left, symmetric, or right-ultrasensitivity behaviors can then be distinguished and associated to \( n_{H}^{L} / n_{H}^{R} \) values larger, equal or lower than one, respectively. In the same way that dose-response curves with symmetric ultrasensitivity are usually described by Hill functions, asymmetric ultrasensitivity functions could be adjusted by a piecewise Hill function,

\[
R(x) = \frac{x dy}{y dx} = \frac{d \log y}{d \log x}
\]

(3)

The response coefficient estimates the local polynomial order of the response, providing a local description of relative changes of input (x) and output (y) levels.

As shown in figure S1 for several examples, quantities such as the polynomial order of the response function (equation (3)) and/or sensitivity asymmetries (equation (2)) can be used to unveil and quantify differences between diverse ultrasensitive responses. As we will discuss below, these differences in local sensitivity features are extremely relevant and have noticeable consequences when dynamic range constraints are considered on a given module.

3. Upstream and downstream restrictions

Sigmoidal response modules may be found in many different cellular contexts, integrated with diverse upstream and downstream components. In this section we present a simple setup designed to quantify how the sensitivity of the combined system is affected by constraints imposed on the effective input and output dynamic range of the ultrasensitive module (see figure 1).

In order to focus our analysis on effects exclusively due to dynamic range restrictions on the analyzed ultrasensitive unit, hyperbolic responders were chosen as upstream and downstream components of the three module system.

Transfer functions like the ones considered in our analysis present two characteristic concentration scales: one associated to its EC50 value and the other to its maximum response level, \( O_{\text{max}} \). As can be easily appreciated from figure 2(a), both quantities provide natural scales that may determine the ultimate behavior emerging from the interaction between the ultrasensitive module under analysis and its input and output (readout) components. The interplay between these relevant concentration scales of consecutive components can be described by upstream, \( Q_{\text{up}} \), and downstream, \( Q_{\text{down}} \) restriction coefficients, defined as follows:

\[
Q_{\text{up}} = \frac{EC50_{M}}{O_{\text{max}, \text{up}}} \quad Q_{\text{down}} = \frac{O_{\text{max}, M}}{EC50_{\text{down}}}
\]

(4)

where \( O_{\text{max}} \) represents the maximum output level and \( M \), up and down subscripts refer to parameters of the ultrasensitive module, upstream component and downstream component respectively.

For small values of \( Q_{\text{up}} \) the full input dynamic range of the ultrasensitive module is explored by the output generated by the upstream component. Similarly, for small values of \( Q_{\text{down}} \) the saturation of the downstream component occurs at output concentrations well beyond the central module’s maximum output. Hence, when both, \( Q_{\text{up}} \) and \( Q_{\text{down}} \) display values much lower than one, the interaction of the upstream and downstream components with the sigmoidal central module is linear and the overall system’s sensitivity is expected to be the same as that of the central module in isolation (left panel of figure 2).

On the other hand, large values of the upstream restriction coefficient \( Q_{\text{up}} \) imply that the central module’s EC50 is much larger than the maximum output concentration produced by the upstream component. As can be seen from the example depicted in the center panel of figure 2, a severe...
reduction in the central module’s effective working range (i.e. the range of inputs that would actually stimulate it) could take place when $Q_{\text{up}} \gg 1$ and $Q_{\text{down}} \ll 1$. In this case, we will say that the module is in an upstream saturation or upstream limited regime. Considering the toy model introduced in figure 1(b), this would correspond to a situation in which the upstream module’s maximum response ($O_{\text{max,up}} = |B|_1$) activates only a small amount of $[C]$. In this case, $O_{\text{max,up}}$ would be lower than the input needed by the ultrasensitive module’s to achieve its half maximum response (i.e. $O_{\text{max,up}} \ll EC_{50,up}$) resulting in $Q_{\text{up}} \gg 1$.

Finally, large values of the downstream restriction coefficient, $Q_{\text{down}}$, correspond to setups where the central module’s maximum output is much larger than the EC50 of the downstream component. In cases like the one depicted in the right panel of figure 2, where $Q_{\text{down}} \gg 1$ and $Q_{\text{up}} \ll 1$, the range of different outputs of the central module cannot be fully ‘read’ by the downstream unit, and thus its readout range could be severely limited. In this case we will say that the module is in a downstream saturation or downstream limited regime. In the presented toy model this would occur whenever a small amount of $[C^*]$ is enough to fully activate $[D]$. This scenario will corresponds to the case in which the maximum module’s output is much higher than the ultrasensitive module’s input needed to achieve the half maximum response ($O_{\text{max,M}} \gg EC_{50,down}$) and hence $Q_{\text{down}} \gg 1$ (a quantitative characterization of upstream and downstream saturation regimes is provided in the supplementary material SM2 for the case of a Hill module).

4. Analysis of constrained ultrasensitive modules

In either downstream or upstream saturation scenarios, input and readout processes can constrain the explored dynamic range of the sigmoidal module, making local sensitivity features of the analyzed transfer function become relevant to understand the overall system behavior. To illustrate this point, in this section we present a systematic analysis of the sensitivity of generic ultrasensitive responders coupled to input and readout hyperbolic modules, under different dynamic range restriction conditions. We start by analyzing the case where the ultrasensitive central module’s response function is described by a Hill-type mathematical form. We then proceed to extend the analysis to several ubiquitous ultrasensitive motifs, whose ultrasensitive transfer functions can be derived from the corresponding mechanistic models.

4.1. Hill-type modules

The use of Hill mathematical forms to fit experimental data is a fairly common practice. It is then worth studying the impact
of input and readout dynamic range restrictions in this case (without loss of generality we will consider an \( n_H = 3 \) ultra-sensitive Hill module).

We start our analysis writing down the expression of the overall system’s output as a function of the input signal, \( O(I) \), calculated through a simple mathematical composition of the respective single-module transfer functions:

\[
O = \frac{O_{\text{max,down}}}{Q_{\text{down}} + 1} \left( 1 + y^{\kappa n} \right) + y^{\kappa n} = \tilde{O}_{\text{max,down}} \left( 1 + y^{\kappa n} \right)
\]

where \( \tilde{O}_{\text{max,down}} = O_{\text{max,down}} \frac{Q_{\text{down}}}{Q_{\text{down}} + 1} \), \( \kappa = \frac{Q_{\text{up}}}{\sqrt{1 + Q_{\text{down}}} \right. \)} and 

\[
y = \frac{I_{\text{up}}}{EC50_{\text{up}}} \text{ is the input signal of the upstream hyperbolic module (i.e. the input signal for the entire system).}
\]

Using equations (5) and (1) we obtained the following analytical expression for the system’s Hill coefficient, \( \eta \):

\[
\eta(q_{\text{down}}, Q_{\text{up}}, n_H) = \frac{\log 81}{\log \left( \frac{y_90}{y_{10}} \right)}
\]

\[
= \frac{\log 81}{\left( \frac{10 + 9k^{-n_H}}{9 + k^{-n_H}} \right)^{1/n_H} - 1}
\]

where \( y_{10} \) and \( y_{90} \) are the \( y \) values which produces the 10% of \( \tilde{O}_{\text{max,down}} \) and 90% of \( \tilde{O}_{\text{max,down}} \) in equation (5).

Figure 3(a) shows the dependency of the system’s overall sensitivity \( \eta \) on parameter \( \kappa \) given by equation (6). It can be observed that increasing \( \kappa \) results in a loss of the system sensitivity, up to a point where for \( \kappa \geq 1 \), the range-constraint...
The effect is fully developed and the global system achieves its minimal sensitivity. Noticeably, for \( \kappa \ll 1 \) the global sensitivity equals that of the central Hill module.

Equation (6) reveals that for this particular system the effect of upstream and downstream restrictions on the system sensitivity is in fact a function of a single parameter, \( \kappa \). Noticeably, this parameter is proportional to the \( Q_{up} \) restriction coefficient, but decreases with increasing \( Q_{down} \) values. In this way, for this kind of modules, upstream restrictions degrade the module sensitivity level whereas downstream restrictions tend to preserve it. In order to further analyze this result it is worthy to describe the system behavior as a function of \( Q_{up} \) and \( Q_{down} \) coefficients. In addition this would allow us to establish meaningful comparisons with the analysis of other ultrasensitive motifs, for which an analytic expression cannot be easily obtained.

In figure 3(b) we present a color-coded systematic characterization of the Hill module’s effective sensitivity, numerically estimated using equation (1) as a function of \( Q_{up} \) and \( Q_{down} \) coefficients. A \( \kappa (Q_{down}, Q_{up}) = 1 \) line was included in the panel (gray line) to delineate the (upper) area of severe ultrasensitivity reduction.

It can be observed that in the absence of dynamic range restrictions (\( Q_{down} \) and \( Q_{up} \ll 1 \), bottom-left area of figure 3(b)) the system’s estimated sensitivity equals that of the central module. This is the expected behavior from figure 3(a) as in this case \( \kappa \ll 1 \).

On the other hand, as expected from equation (6) and the \( \kappa \) dependency on \( Q_{up} \) and \( Q_{down} \), parameters, it can be recognized from figure 3(b) that upstream and downstream constraints have different effects on the system behavior. For a given \( Q_{down} \) level, a clear diminution of the system’s sensitivity can be observed for setups displaying increasing values of the upstream restriction coefficient \( Q_{up} \). On the contrary, for a given \( Q_{up} \) value, a rise in the downstream restriction coefficient value \( Q_{down} \), corresponds to a decrease in the \( \kappa \) parameter level, and results in an increase system’s sensitivity levels.

### 4.1.1. Analysis of highly constraint situations.

Analytical expressions for the overall response function can be obtained for highly constraint situations (i.e. when \( Q_{up} \gg 1 \) and/or \( Q_{down} \gg 1 \)). The dynamic range effectively spanned by the Hill’s module when input and/or readout saturation takes place in this system is actually limited to the module’s low input region (see supplementary material SM3, and figure 2).

In any of these saturation scenarios the central module response function can be approximated by:

\[
O_M = O_{max} \frac{I_M^{up}}{EC50_M^{up}} + I_M^{up} \sim O_{max} \frac{I_M^{up}}{EC50_M^{up}} \quad (7)
\]

with \( I_M \), the input signal of the analyzed central module \( M \).

As we show in the supplementary material SM2, when equation (7) is a valid approximation, the readout unit restricts the module’s effective working range whenever \( Q_{down} \gg Q_{up}^{*} \). In this case the condition \( \kappa \ll 1 \) is satisfied and no sensitivity reduction is expected to take place in the system. Accordingly, for situations where the downstream component’s output reaches saturation, and the upstream module works in its linear regime (i.e. \( I_M \) spans the linear region of the upstream module’s transfer function, see supplementary material SM2 for mathematical details) the input unit’s response function can be approximated by \( O_{up} (I_M) \sim O_{max} I_M / EC50_{up} \), and the whole system transfer function results in

\[
O_{down} (O_M (O_{up} (I_M))) \sim O_{max} \frac{I_M^{up}}{\sigma^{\eta n} + y^{\eta n}} \quad (8)
\]

where \( y = \frac{I_M}{EC50_{up}} \) and \( \sigma = \frac{Q_{up}}{Q_{down}} \). Equation (8) shows that under this regime, when it is the readout module the only one that saturates, the system responds as a Hill function with \( \eta = n_H \). The analyzed situation corresponds to the right-bottom corner of figure 3(b) where, as expected, the numerically estimated \( \eta \) value was found to be close to \( n_H \).

On the other hand, saturation takes place at the upstream unit whenever \( Q_{up} \gg \sqrt[\eta]{Q_{down}} \) (see supplementary material SM3 for mathematical details).
SM2). This corresponds to \( \kappa \) getting values larger than one, and the system is expected to achieve its minimal sensitivity level (see figure 3(a)). In this case the upstream module saturates and the downstream readout module, on the contrary, operates nearly in the linear regime, so we can speak of an upstream saturation regime. The response function of the downstream readout module can be approximated by \( O_{\text{down}}(I_{\text{down}}) \sim O_{\text{max}} \downarrow I_{\text{down}}/EC_{50} \downarrow \), and the entire system transfer function can be expressed as

\[
O_{\text{down}}(O_M(O_{\text{up}}(I_{\text{up}}))) \sim \frac{O_{\text{max}} \downarrow}{\sigma^{nH}} \left[ \frac{y}{1 + y} \right]^{nH} \tag{9}
\]

Equation (9) does not have the general Hill functional form. Instead, powers of \( y \) of orders between 0 and \( n \) now appear in the denominator, resulting in a drop of the system’s effective sensitivity, in concordance with the behavior expected from figure 3(a). This working regime corresponds to the entire upper area in figure 3(b), where the condition \( Q_{\text{up}} \downarrow \gg Q_{\text{down}} \) holds (see supplementary material SM2 and SM4).

### 4.2. The general sigmoidal case for constraint situations

The above-presented analysis may be easily generalized to sigmoidal response functions that, as the Hill mathematical function case, could be approximated in the low input signal range (i.e. in the input’s range actually covered under a downstream/upstream limited situation) by

\[
O_M \sim A^2 \mathrm{I}^n_H \tag{10}
\]

Here \( n \) is a positive constant that equals the mean polynomial order of the transfer function for small input signals, and \( A \) is a proportionality constant.

Whenever equation (10) holds, equations (8) and, (9) valid for downstream and upstream saturation situations respectively, can be recast into the following expressions:

\[
O_{\text{down}}(O_M(O_{\text{up}}(I_{\text{up}}))) \sim O_{\text{max}} \downarrow \frac{\sigma^{nH}}{\sigma^{nH}} \left[ \frac{y}{1 + y} \right]^{nH} \tag{11}
\]

\[
O_{\text{down}}(O_M(O_{\text{up}}(I_{\text{up}}))) \sim \frac{O_{\text{max}} \downarrow}{A EC_{50} \downarrow} \left[ \frac{y}{1 + y} \right]^{nH} \tag{12}
\]

It is worth noting that it is the mean polynomial order exhibited at low input ranges, \( n \), and not the Hill coefficient, \( n_H \), of the analyzed transfer functions, what affects the system’s effective sensitivity value.

Equation (11) states that the overall system response is of the Hill function mathematical form, but importantly, it has \( \eta = n \) (and not \( n_H \)) as its Hill exponent. Importantly, this means that downstream saturation could produce super-multiplicative behavior for transfer functions which present low-input polynomial order values, \( n \), larger than the overall displayed ultrasensitivity, \( n_H \) (i.e. satisfying \( n > n_H \)).

### 4.3. Goldbeter-Koshland modules

The term ultrasensitivity was originally coined by Goldbeter and Koshland (GK) to describe amplification sensitivity capabilities displayed by covalent modification cycles, such as phosphorylation-dephosphorylation (Goldbeter and Koshland 1981) (see figure 4). For these systems, GK derived an analytical expression of the corresponding response function (i.e. the GK response function) valid when enzymes are in much lower concentration than their substrates. Moreover, they showed that a substantial increase of the system’s sensitivity can be observed when both, kinases and phosphatases, are saturated. Given the prominent role played by this motif as a building block of signaling cascades, this way of generating ultrasensitivity—termed zero-order ultrasensitivity mechanism—has received a lot of attention (Ferrell 1996, Racz and Slepchenko 2008, O’Shaughnessy et al 2011).

![Figure 4. Goldbeter and Koshland covalent cycle. (a) Scheme, (b) biochemical reactions.](https://example.com/figure4.png)
In order to analyze the effect of dynamic range limitations on this important motif, we considered a GK ultra-sensitive module where both, kinase and substrate, were equally saturated (without loss of generality we fixed the system’s parameters to achieved an \( n_H = 3 \)). For this ultra-sensitive module we proceeded to numerically estimate using equation (1) the overall system’s sensitivity, \( \eta \), in different upstream and downstream constraint scenarios (figure 5(a)).

As in the above analysis, when input and/or readout saturation effects were noticeable, the module’s effective working range was constrained to its low input signal region (see figure 2). However, in this case, the polynomial order of the GK transfer function tends to \( n = 1 \) for low enough input signals (see figure S1b). As a consequence, there is now a loss of sensitivity not only for upstream limited regimes (upper area of figure 5(c)) but also for large values of \( Q_{down} \) in the downstream limited regime (right-bottom region of figure 5(c)).

It is interesting to consider the case of asymmetric GK ultrasensitive functions, where only one of the enzymes is saturated (i.e. either the phosphatase is saturated and not the kinase or the kinase is saturated and not the phosphatase), producing left or right-ultrasensitive response curves (see supplementary material SM1). Right and symmetric cases exhibit no differences in their qualitative behavior (figure 5(b)). However noticeable differences arise in the effect of constraints on the central module’s dynamic range for the case of a left-ultrasensitive GK function (figure 5(c)). As for the symmetric case, a loss of sensitivity can be observed for upstream limited regimes (upper area of figure 5(c)). However, for intermediate downstream saturation levels in absence of upstream saturation (bottom-center in figure 5(c)) a strong increase in sensitivity can be observed. In this regime, the readout range encompasses the most sensitive part of the left-ultrasensitive curve, increasing the effective system’s sensitivity. For higher values of the downstream restriction coefficient, the module’s effective output dynamic range is further reduced, the polynomial first order nature of the response becomes dominant (\( n = 1 \)), and the system’s sensitivity is severely lost reaching \( \eta = 1 \) (bottom-right in figure 5(c)).

4.4. Multistep activation modules

In a multistep activation module, a common input signal simultaneously regulates more than one biochemical process that synergistically produces an output response (Zhang et al 2013). These motifs are relevant in many biological situations, for instance for multiple distributive phosphorylation processes (Gunawardena 2005) or in the context of gene regulation, when the promoter region of a transcription factor’s target gene has multiple binding sites (Rippe 1997).
We analyze here an instance of the latter scenario, but equivalent conclusions could be drawn for multiple phosphorylation systems in the limit of non-saturated reactions (Gunawardena 2005).

We proceed by considering the biochemical scheme of reactions taking place inside the transcriptional module shown in figure 6, where, for the sake of simplicity, we assumed that there are only two cis-regulatory response elements. In this scheme A is the transcription factor, and B the associated transcribed mRNA.

Assuming mass action kinetics, the steady state module’s transfer function can be written as:

$$ O_M(A) = \frac{1}{\delta} \frac{v_1 K_2 A + v_2 A^2}{K_1 K_2 + K_2 A + A^2} $$

where $K_i = k_i^- k_i^+$ is the equilibrium dissociation constant for the $i^{th}$ binding site. In the case where $v_1 = 0$ (i.e. protein transcription can be neglected for single-site binding) the module can achieve the maximal sensitivity value, $n_H = 2$, in the limit of $K_1 \gg K_2$, which corresponds to high cooperativity between binding sites (Rippe 1997). On the other hand, for $K_1 \ll 1$ the response tends toward a hyperbolic functional form presenting $n_H = 1$.

It is worth noting that the polynomial order (i.e., the response coefficient) of the transfer function given by equation (13) in the low input region is quadratic. As we have described above, this implies that under a downstream restriction regime, this module could achieve an effective sensitivity of $\eta \sim 2$. Notably, this arises directly from a structural property of the module, independently of the particular choice of its internal parameter values. This means that even in the case of no-cooperativity (for instance, if $K_1$ and $K_2$ were of the same order) maximal ultrasensitivity may be achieved in the regime where the readout module saturates. We show in figure 7(a) the transfer function corresponding to equation (13), for a choice of internal parameter values compatible with a module sensitivity level of $n_H = 1.36$. The response function presents a left-ultrasensitive character (with $n_{HI} = 1.55$ and $n_{HI}^R = 2.1$). In fact, for low input signals the module can display sensitivity levels larger than $n_H = 1.36$ ($n = 2$), and consequently the overall system’s sensitivity could increase under downstream restrictions. To illustrate
Figure 8. Simple model of protein sequestration and transcription. Active transcription factor A is sequestered by inhibitor B into an inactive AB complex that cannot bind DNA. (a) Scheme, (b) biochemical reactions.

this point, in figure 7(b) we show the analysis of the three module system’s sensitivity. As expected, it can be appreciated that for large values of the downstream restriction coefficient, a sensitivity level of $\eta = 2$ can be achieved, similarly to what it could have been obtained from an isolated module displaying high levels of cooperativity.

4.5. Competition modules

Molecular titration and stoichiometric inhibition processes can lead to left-ultrasensitive behavior if an inhibitor B is abundant and has high affinity for a signaling species A (Ferrell 1996, Buchler and Louis 2008, Rowland et al 2012). At low values of total A, all of it is in the form of an inactive complex B:A. This will be true up to the point where all B is consumed by the complex. At this threshold point all the buffering capacity of B is exhausted and a small signal increment, $\Delta A$, will be almost entirely available for activating a downstream target, leading to a sizeable increase in the response function. As an example of this type of ultrasensitive motif we studied a simple model of protein sequestration and transcriptional response proposed by (Buchler and Cross 2009) (see figure 8).

According to the Buchler and Cross model (see supplementary material SM5) the equilibrium concentration of a transcription factor A, that can reversibly bind to a stoichiometric inhibitor B, can be expressed as:

$$A = \frac{A_T - B_T - K_d + \sqrt{(A_T - B_T - K_d)^2 + 4 A_T K_d}}{2}$$

where $A_T$ and $B_T$ are total concentrations of the signaling and inhibitor species, respectively. $K_d = k_i^d / k_i^+ = k_i^d / (k_i^d + k_i^+)$ is the reaction dissociation constant, given as a ratio of the backward and forward kinetic constants, $k_i^d$ and $k_i^+$, respectively. In addition, A can also bind to a single DNA-binding site, activating a transcriptional response at a rate $O$. If basal transcription can be disregarded, the steady state system’s transcriptional response is given by

$$O = O_{\text{max}} \frac{A}{\epsilon + A}$$

where $\epsilon = k_i^d / k_i^+$ is the EC50 of the hyperbolic response function and $O_{\text{max}}$ is the maximal induced mRNA transcription rate (see supplementary material SM5 for details).

We show in figure 9 the corresponding module response function, $O = O(A_T)$, for a case where the buffering effect of B is expected to be sizable ($K_d / B_T \ll 1$). The input-output curve has a marked threshold for $A_T \sim B_T$ (around a value of 100 in figure 9(a)). The module’s transfer function is strongly left asymmetric ($\eta_B = 2$, $\eta_{A,B} = 3.7$, $\eta_{A,B} = 1.4$). This implies that if a downstream readout module imposed constraints on the effective dynamic range, the left-ultrasensitive character of the module response function could be exploited to obtain a large sensitivity for the complete constrained system.

It is important to point out that the left Hill coefficient is also a global measure, and might not reflect the existence of much higher levels of local sensitivity (see supplementary material SM1). This is indeed the case here, and the reason why, even though the left Hill coefficient is only 3.7, an overall effective sensitivity of about 12 may be obtained for this system in a downstream restriction regime. This is reported in figure 9(b), where up to a six-fold global sensitivity increase can be observed for mild downstream limited regimes (bottom-center region of figure 9(c)).

4.6. Arrangement of two covalent cycles

In this section we aim to illustrate how the concepts introduced so far could be used to analyze more general setups in which dynamic range misalignments could also play a major role in connection with the ultrasensitive character of the system.

Slepchenko and collaborators showed that linear arrangements of covalent cycles acquire a Hill sensitivity that exceeds the multiplicative limit for situations where activators are much less saturated than inhibitors (Racz and Slepchenko 2008). In this regime, sequestration effects are negligible, and we can use our general framework to analyze the supra-multiplicative behavior they reported. Moreover, it is worth noting that in these asymmetrical configurations the corresponding transfer functions present a markedly left-ultrasensitive character suggesting that the observed increase in sensitivity could be explained in terms of read-out dynamic range restrictions operating on the composed system.

In order to evaluate this possibility, we numerically generated an ensemble of asymmetric bicyclic covalent cascades using the same parameter sampling procedure used by Slepchenko in (Racz and Slepchenko 2008). For each cascade instance we considered the second module as the read-out unit of the first one, and estimated the corresponding downstream restriction coefficient values, $Q_{\text{down}}$ (see supplementary material SM6 for details). In addition, we assessed the observed supra-multiplicative character of the system defining the oversensitivity coefficient: $\pi = n_{\text{cycle}}^\text{cascade} / (n_{\text{cycle}}^\text{cycle})^2$, which is larger, lower or equal to 1 when the two cycles combines supra-multiplicatively, submultiplicatively or multiplicatively respectively. For the sake of completeness, relevant features of the considered model, and of the sampling procedure are summarized in the supplementary material SM6.

We found (green points in figure 10) that supra-multiplicative behavior ($\pi > 1$) occurs for almost every cascade realization when the analysis was constrained to exactly the same subspace of parameters (see supplementary material...
SM6 for details) that was explored by Slepchenko and collaborators in (Racz and Slepchenko 2008). It can be recognized from the figure that the sampled realizations spanned mild downstream restriction coefficient values. When we relaxed some of the constraints of the original sampling methodology (see supplementary material SM6) a wider interval of \( Q_{\text{down}} \) could be spanned (blue points in figure 10) revealing a non-monotonous behavior of \( \pi = \pi(Q_{\text{down}}) \).

The observed peak in the oversensitivity is entirely compatible with the behavior reported for mild downstream limited scenarios found in the two downstream constraint left-ultrasensitive response functions: asymmetric GK cycles and competition modules (see bottom part of figures 5(c) and 9(b)). These findings suggest that the behavior of the overall sensitivity level of the compound system could be understood in terms of the downstream restriction concepts introduced in this paper.

5. Discussion and conclusions

Despite the success of module-based approaches to disentangle the complexity of cellular biochemical networks, on a general basis, care should be taken when realistic, integrated systems are analyzed, to accommodate the cases where the properties of individual components change upon interconnection.

In the present contribution we asked if the simplest possible non-trivial form of distortion by interconnection, i.e. limitations on the input and/or output effective working ranges of a given module imposed by upstream and downstream components, could produce substantial changes to the ultrasensitive features of a sigmoidal module.

We made use of a three-module setup, and considered the case where different ultrasensitive modules of interest were coupled to Michaelis-Menten-type modules, acting as input and read-out components. We evaluated the overall system sensitivity considering, for each analyzed case, different working-range misalignment situations assessed in terms of upstream and downstream restriction coefficients, \( Q_{\text{up}} \) and \( Q_{\text{down}} \).

We found that, independently from the detailed mechanism involved, whenever the upstream module acts as the limiting component (i.e. large \( Q_{\text{up}} \) values), the global
ultrasensitivity character of the system is severely reduced. On the other hand, we found a much richer dependency of the overall system’s ultrasensitivity on the downstream restriction coefficient, $Q_{\text{down}}$. Whenever the downstream unit is the limiting element of the cascade (i.e., for large $Q_{\text{down}}$ values, and small $Q_{\text{up}}$ values) different behaviors arise depending on the specific non-linearities displayed by the analyzed ultra-sensitive module (GK versus Hill, for example).

In the light of these results, downstream dynamic range restrictions should be particularly taken into consideration when analyzing modules presenting left-ultrasensitive responses. We already analyzed molecular titration modules based on a threshold model of transcription presented by Buchler and Cross (Buchler and Cross 2009). In a recent analytical study, Gunawardena found that ordered distributive multisite phosphorylation systems could also have response functions of the same mathematical form than the one depicted in figure 9(a) (compare this figure with figure 2 in Gunawardena’s work (Gunawardena 2005)). He reasoned that this kind of transfer function creates an efficient threshold. The system response is maintained close to 0, for input signals below a suitable threshold ($\text{input} \leq 100$ in the case of figure 9). However, above the threshold, the response does not switch between 0 and 1 abruptly, as would be the case for an efficient switch. In this context, it is worth noting that, even though such a module could be considered a bad switch when working in isolation, a downstream-limited readout unit could easily convert it into a good switch taking advantages of the local non-linearities of the module’s response curve, as was demonstrated in figure 9(b) for the competition module.

In addition, our general framework allowed us to analyze distinctive features displayed by specific biochemical motifs. For instance we noticed that, irrespectively of their specific degree of cooperativity, multistep activation modules could produce high levels of sensitivity under downstream limited regimes. Interestingly, when left-ultrasensitive transfer functions were involved, the system overall ultrasensitivity could be significantly enhanced for relatively mild downstream constrained situations. For these cases, one may imagine that the downstream component’s EC50 value is fine-tuned, in the sense that only the most sensitive part of the sigmoidal module’s output is read by the downstream unit just before it reaches its saturation levels. In this way, downstream saturation could be considered as a new mechanism on its own, capable of building a highly ultrasensitive device, from an original asymmetrical and modestly ultrasensitive module.

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