Introduction to biological complexity as a missing link in drug discovery

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Introduction

Despite a burgeoning knowledge of the intricacies and mechanisms responsible for human disease, technological advances in medicinal chemistry, and more efficient assays used for drug screening, it remains difficult to discover novel and effective pharmacologic therapies.

Areas covered

By reference to the primary literature and concepts emerging from academic and industrial drug screening landscapes, we propose that this disconnect arises from the inability to scale and integrate responses from simpler model systems to outcomes from more complex and human-based biological systems.

Expert opinion

Further collaborative efforts combining target-based and phenotypic-based screening along with systems-based pharmacology and informatics will be necessary to harness the technological breakthroughs of today to derive the novel drug candidates of tomorrow. New questions must be asked of enabling technologies- whilst recognising inherent limitations- in a way that moves drug development forward. Attempts to integrate mechanistic and observational information acquired across multiple scales frequently exposes the gap between our *knowledge* and our *understanding* as the level of complexity increases. Here we offer our thoughts and some actionable items that we hope will inform directed evolution of the drug discovery process.

Keywords

Drug development
Systems
Complexity
Models
Phenotypic screen
Mechanisms

"Disease is not something personal and special, but only a manifestation of life under modified conditions, operating according to the same laws as apply to the living body at all times, from the first moment until death". Rudolf Virchow (Die Epidemien von 1848, 7)

1. Introduction to complexity as the missing link in drug discovery

Technological innovations and advances continue to provide more detailed and refined mechanistic information on diseases targeted for novel pharmacologic therapies. There has been an explosion in approaches using human cell systems with disease-relevant genotypes and phenotypes (e.g. organ-on-a-chip, stem-cell derived models), advances in chemistry, new automated platforms for high-throughput, high-content screening, numerous 'omics' approaches, and advanced computational tools for visualising and deep-mining data to provide data regarding effects of novel drug candidates [1, 2]. Many of these advances provide enhanced tools for re-running 'traditional assays in new formats, and thus allow us to take full advantage of screening vast compound libraries to derive tomorrow's new drugs. However, we are essentially asking the same questions of these platforms and as a result we continue to identify compounds that, more often than not, fail to translate into clinical utility. So far, we have failed to utilize these new technologies in truly transformative ways along the drug development process.

Part of the problem stems from the use of simpler model systems in which the outputs are easier to interpret and which themselves are more amenable to higher throughput screening approaches but which offer only restricted insights into the disease-causing process and/or the real mechanisms of drug effect. There is still much regarding the multiple levels of organization and complexity in biology and disease that we do not understand. Consider the use of stem cell-derived systems (e.g. embryonic- and induced pluripotent stem cell-derived cardiomyocytes and hepatocytes) increasingly being used in drug discovery. The opinion that they are not the panacea for efficacy or safety studies may be true but they do offer some obvious advantages over non-human equivalents and represent a pragmatic choice of cell platform for studying "disease-in-a-dish" [3, 4, 5]. However, no cell model fully recapitulates all facets of the biology that occurs in vivo (hence the term "models), and it is legitimate to question whether such assays are "fit for purpose". Furthermore, data acquired from these preparations may be prone to misinterpretation if the limitations inherent in the models are not considered (see [6] for a specific example relating to the absence in stem-cell derived cardiomyocytes of a K^+ channel that is critical in establishing human resting membrane potential (IK_1)). Whilst substantial efforts and resources are directed at modifying such cell preparations in order to mimic their behaviour in tissues in vivo, still many extrinsic factors that shape proper cellular form and function are missing from current experimental setups. The precise configurations of heterocellularity and interconnectedness, regional variability within

tissues, mechanical tensions, electrical and hormonal cues, nutrient availability and localised redox environments that may change dynamically in vivo- and which influence steady-state behaviour and biological responsiveness- are extremely difficult to recreate in vitro. Such considerations have shifted the discussion from "how relevant is that mouse study to human findings" to "how relevant are human cell-derived model studies to human findings".

It seems that in our efforts to understand the reasons behind the technical inadequacies of contemporary experimental platforms used in drug discovery we are fundamentally failing to grasp less tangible aspects that are intimately involved in shaping biological behaviour and responsivity. What are those elements that evade easy quantification and which provide missing links enabling us to better translate "data richness" into improved clinical therapies? Here, we present the case that a key link presently missing- and one that is pivotal in moving drug development forward from preclinical models (using non-human cells) to 'proclinical' models (using human-derived cells in preclinical studies) and from there to clinical utility - is *complexity*.

We are not suggesting that drug hunters do not understand or appreciate the intricate complexities involved in normal physiology or diseased states [7]. In this this review, we posit that experimental tools and approaches used to probe complex human systems are imperfect and incomplete and as a consequence give rise to outputs that are difficult to properly synthesise and interpret. In section 2, we illustrate, with reference to examples, the requirement for integrating data from multiple models characterised by different levels of complexity. Section 3 distils our thoughts on some of the factors that influence the ability to properly interrogate the component model systems that are used in building more complex systems. Lastly, Section 4 briefly describes the need to consider the 'physiologic gamut' in order to appropriately contextualise biological range and complexity.

2. The need for multiple models of different complexity

Figure 1 depicts a schematised framework of "quantitative and systems pharmacology" (QSP) that integrates information emerging at multiple scales using the idea of 'horizontal' and 'vertical' network architecture [8, 9]. Erwin Chargaff's prediction in the context of genetic engineering that "If you can modify a cell, it's only a short step to modifying a mouse, and if you can modify a mouse, it's only a step to modifying a higher animal, even man" [10] may hold true conceptually, but it does not fully recognize the nature of all of the components- some quantifiable, some not- involved in 'scaling up'. There is an essential need to understand the basis of the increased complexity associated with transitions from the molecular scale through to organisms and beyond that reflects the fidelity of translation to the clinic; such transitions are

chasms, not short steps and not unlike the “Valley of Death” phrase used to describe the arduous transition of drugs from laboratory findings to successful human clinical trials.

The tension created by considering how best to integrate information acquired at different scales of complexity is mirrored in the general approaches used to screen for new drugs. Swinney and Anthony’s review highlights the need to make two essential elements of the drug development toolkit work together in a more meaningful way to guide drug discovery efforts [11]. The first approach, labelled mechanistic- or target-based screening (TBS), usually involves the detailed characterization of lower-order complexity systems (e.g. molecule or signalling node) to resolve distinct molecular mechanism of action (MMOA). The second approach, labelled phenotypic screening (PS), is defined by Swinney and Anthony as “all modes of assays in which a biological system (and the perturbation therein) can be faithfully recapitulated”. PS seeks to quantify measurable changes in the behaviour of more complex systems in the absence of knowledge of the MMOA and thus describes empirical methods to evaluate integrated responses of biological systems of varying complexity (for example, pathways, cells, tissues, animals). Arguably, PS has played a major role in discovering therapeutics with novel mechanisms of action (so-called first in class drugs) [11, 12]. Such successes have been attributed (at least in part) to preservation of systems complexity in experimental models and the ability to monitor relevant (and often integrated) endpoints. It could also be argued that PS involves more serendipity; others have offered views on how this might be systematized [13].

In general, most drug discovery efforts are enabled by a newly-recognized understanding of a mechanism or pathway that plays a putative key role in a complex disease. For example, it is expected that by modifying a key receptor, enzyme, metabolic pathway, signaling cascade or expressed protein that disease progression is favorably altered or reversed. This ‘target-centric’ approach allows for a linear progression of stage gates that govern a wide range of discovery efforts that include lead identification and optimization, preclinical efficacy and safety testing, pharmacokinetic modeling and formulation, and early phase 1 (first in human) studies leading to subsequent pivotal clinical trials to test for efficacy. This strategy is most likely to succeed if the targeted mechanism a) plays a unique role in human disease progression b) is well understood within cellular, organ, and organism contexts, and c) if either more simplified models (including in silico, in vitro, ex vivo) and more complex models (including animal models) are available that faithfully recapitulate the disease or events leading to the disease. The possible contribution of multiple on-target effects involving less recognized (or unknown) pathways to the overall efficacy of novel therapeutics may further complicate an understanding of a drug’s efficacy. Further, a drug’s off-target (or side-effects) profile at exposures comparable to those defining efficacy may also confound understanding

drug efficacy. It is more likely that phenotypic effects would detect off-target effects (as compared to TBS focused on MMOA).

Unfortunately, most (if not all) of the three assumptions listed above are only partially fulfilled; if we knew everything about the disease, it would be much simpler to design and discovery appropriate chemistries from an ever-expanding list of approaches (spanning small molecules and biologics to delivered gene therapies) to affect pharmacologic endpoints. For practical reasons that include speed, efficiency, cost, and clarity of interpretation, simpler model systems are generally used (and favored) for screening of chemical libraries in early discovery efforts. If available, more complex models may be employed later in drug discovery to more fully evaluate efficacy (if such disease models exist) and safety (using non-diseased models). Absent of such models, biomarkers may be used as surrogate markers for efficacy, informing on target engagement. These same biomarkers may also be used in early human studies to again test for target engagement, an essential first step in validating a therapeutic target in clinical studies.

More recently, PS have been applied to safety studies using humanized models (e.g. human induced pluripotent stem cell-derived cardiomyocytes and hepatocytes [4, 5]), with the potential advantage that the human-derived biology under study more closely represents the clinical experience. Patient-derived stem cell-based in vitro models (for either safety or efficacy studies) have also been described in which simpler human derived cells, or more complex co-cultures, and organoids are used. A major question for these “disease in a dish” studies is the extent to which the disease phenotype is faithfully (and reproducibly) replicated. This is more likely to be achieved with monogenic inheritable diseases than with diseases resulting from multiple genetic and environmental influences. More recent gene-editing tools provided by the discovery of CRISPR-Cas techniques hold promise for creating more complex humanized in vitro disease models provided that we understand better the underlying disease mechanisms. Of course, a fusion of both target- and phenotypic-directed experimental approaches can be used, depending on the understanding of the complex biology and our ability to recreate and monitor complex biological responses.

A principle feature of PS is that the approach affords unbiased assessments of compound activity on a pre-defined aspect of system behaviour (e.g. reduced incidence of seizure or episodes of arrhythmia). However, one of the drawbacks of PS is that because the assays are often necessarily more observational in nature, teasing out the underlying mechanism(s) may be difficult and prone to flawed interpretation. The very nature of PS means that:

1) since any change likely involves networks and interactions between numerous components, the measured outcome may not be directly related to modulation of the presumed molecular target and

2) any outcome may not be predictable from an understanding of the individual components involved (i.e. emergent behaviour).

Such features can cause issues when retrospectively fitting mechanism (i.e. MMOA) to the phenomenological effects observed (i.e. the apparent MOA) in more complex systems. From a more pragmatic perspective, detailed knowledge on the MMOA may be unnecessary when screening drug candidates.

To illustrate the difficulties in reverse-engineering data obtained from higher complexity systems, we consider the histories of three clinically used cardiovascular drugs. Investigations on ranolazine and perhexiline, drugs that were originally developed as anti-anginal agents based on a blueprint of synthetic blockers of voltage-gated ion channels in the cardiovascular system [14], were founded on a presumed mechanism of action that involved direct alterations in metabolic substrate utilization [15, 16]. The endpoints of these clinical studies demonstrated the therapeutic benefit of the drugs under test and the data acquired from NMR on substrate utilization in situ was compatible with a mode of drug action on cellular metabolism. However, in vitro studies in lower complexity systems revealed that the MMOA of ranolazine and perhexiline (with clinically relevant exposures) most likely occurred through voltage, frequency and tissue-dependent block of multiple ion channels involved in cardiovascular homeostasis [14, 17, 18]. Any effect on metabolism would be secondary to the inhibition of these ion channels. We draw particular attention to perhexiline since its serves to highlight the potential for ascribing clinical efficacy to uncertain MMOA if 'single mode' assessments are considered in isolation from data obtained from other experimental platforms. Any action of perhexiline on cellular metabolism (i.e. potent inhibition of carnitine palmitoyl transferase (CPT-1) [16]) was informed by investigations using an *in vitro* biochemical assay of CPT-1 inhibition in rat liver and heart homogenates [19]. Outputs from this assay cannot resolve the contribution of the drug's interaction with other (more well-known) targets *in vivo* (e.g. ion channels) and extensive scrutiny from a medicinal chemistry perspective concluded that the clinical efficacy of this drug is unlikely to be primarily related to an inhibitory action on CPT-1 activity [14, 20]. In another example of the potential disconnection between therapeutic benefit and presumed MMOA, investigations on flecainide, a class I_c (Na^+ channel blocking) anti-arrhythmic that is highly effective in the clinical management of genetic and idiopathic arrhythmia syndromes [21, 22], have produced data that prevent the unequivocal assignation of its MMOA [23, 24, 25]. To reconcile discrepant findings, a 'triple mode' mechanism of flecainide action has been proposed that considers direct and indirect effects of the drug on multiple targets (Na^+ channel, sodium/calcium exchanger and ryanodine receptors) [26, 27, 28]. However, the harmonization of experimental data to fit to a 'unifying' MOA of flecainide has been precluded by findings from single molecule studies (i.e. an exemplar TBS) that contradict any direct effect on ryanodine receptors [29, 30, 31]. This narrative serves to emphasise the potential utility of lower-

complexity systems to specifically resolve the nature of drug-target interactions. These examples highlight the contributions of naming and identifying specific "on-target" actions without considering the full universe of possible drug effects either on individual key cellular components, or the consequences of such interactions across multiple networks and systems within and beyond cells (Figure 1).

The above examples attest to the difficulty of resolving MMOA from studies using more complex systems but also reveal the potential for being unable to delineate between 'cause and effect' by using systems of reduced complexity, especially if the data is considered in isolation from those emerging from complementary approaches. To this end, there is much that can be learned from considering biology using the same principles of interdependency and interconnectedness that are inherent in other network structures. We expand on this in sections 3 and 4, below. The situation with biology (as compared to engineering with mathematical models, for example) is rendered more difficult by practical limitations; the success of resolving MMOA from rather observational effects is largely dependent on the availability of good quality reagents to probe the underlying biology (e.g. antibodies, specific antagonists etc.) and an understanding the networks involved. New approaches that decompose complexity show promise in resolving the direct or consequential actions of a compound on the observed changes [32] and should help mitigate errors of interpretive bias in data emerging from complex platforms.

Although we focus on the need to consider complexity (mindful of the problems associated with doing so), we are not advocating an "anti-reductionist" agenda. After all, the study of very low complexity systems (e.g. flecainide interaction with single ryanodine receptors [29, 30, 31]) is required to unequivocally resolve fundamental mechanisms of drug-protein interaction separate from any confounding complexity (i.e. the true MMOA). Thus, TBS and PS are not 'either-or' options and there is an absolute need for the appropriate placement of TBS in the development pathway. However, the connection between TBS and PS cannot be viewed as a 'linear' link in which one approach naturally follows the other; the deployment of these approaches should be matched to a given phase of the development pipeline and that the level of complexity is tractable and fit for purpose. There is likely to be a need to revisit the approach again once one is armed with more knowledge gained from studies on models of different complexity. We propose therefore to define a 'next generation' of screening frameworks thus: *"complementary assays performed on systems of sufficient complexity that can resolve the contribution of discrete components to mechanism of action in vivo"*. Such capability will inevitably involve computational biology and require informatics support [33]. We consider these issues and other features of future frameworks that will be needed to fulfil these criteria in sections 3 and 4.

3. Exploring dynamical changes in system deterioration

The ultimate goal of pre-clinical assays is to accurately predict a drug's clinical therapeutic efficacy and safety. As Swinney notes, "the more relevant the system is to *physiology*, the better it will predict the clinical success" [12]. The use of the term 'physiology' here is key; physiology represents the functional coalescence of interlocking biological processes in a network architecture that involves the multi-layering of components (Figures 2 and 3). In this context, the prediction of outcome in response to a given drug treatment or pathological disruption is difficult since the network gives rise to a number of characteristics that define the non-linear behaviour of the linked processes (e.g. entrainment, emergence, resilience, robustness, fragility) [34, 35, 36, 37, 38, 39, 40, 41, 42]. It is not practically possible at present to take into account and measure all of the variables and connections that contribute to such system non-linearity. In Figure 2, we use the example of β-AR signalling to illustrate a process where the output (i.e. downstream phosphorylation of multiple proteins) is dependent on multiple network connections. Figure 3 depicts how the release of Ca^{2+} from the sarcoplasmic reticulum (SR) through intracellular Ca^{2+} release channels (ryanodine receptors) is enmeshed in the process of excitation-contraction coupling (ECC) [43] which is regulated by the alignment of 'horizontal' and 'vertical' network elements. We return to the issue of network alignment in section 4.

In terms of attempting to untangle these networks in order to establish cause and effect, Verspignani paints a rather nihilistic picture of "complex systems for which it is generally impossible to abstract the global behaviour from the analysis of single components, especially under conditions of failure [and disaster]" [42]. One of the more paradoxical aspects of physiological complexity is that it is underpinned by the use of comparatively few core components. Akin to a chef producing an array of food dishes from few key ingredients, complexity stems from differential configurations of macromolecular nodes, signalling modules and functional outputs [44, 45, 46, 47]. Given the network constraints imposed on the functionality of each of these interlocked components therefore, it is important to recognize that a fundamental limitation of those studies that model change (e.g. drug-induced toxicity) in more complex physiological systems where redundancy of components is also a key characteristic is that the *most* critical linkages between components are those that *cannot* change if the system is to remain operational. Under these circumstances any measureable change may only *directly* reflect the consequential system adaptation and only *indirectly* the primary causal perturbation.

The notion that signalling components (e.g. G-protein coupled receptors) exist in different physical/functional states in normal and diseased states gives credence to those approaches that aim to assess drug effects only in the diseased state [11]. It is widely

appreciated that drugs developed using 'normal' models may fail to produce anticipated outcomes in the diseased state. Whilst it is clear from our arguments above that it is difficult to develop experimental systems that mimic faithfully such disease-linked reconfigurations in such a way that we can fully understand the model outputs, there is presently huge efforts focussed on biased agonism of cell-surface receptors and in engineering functional receptor selectivity in drug binding studies [48]. Finally, it is essential to conduct preclinical studies in non-diseased models, as most drugs (cytotoxic oncology drugs being the major exception) are administered to normal individuals in early clinical safety studies (before putting patients at risk), and (absent personalized medicine) patients without the disease indication will likely encounter these drugs in clinical settings.

Extending the concepts above, it is necessary to consider the *dynamic* component of pathological processes and disease. It is legitimate to question the relevance of relatively *static* assay modalities. Indeed, one would likely expect different responses treating patients with different severity of disease in different settings (as well as different comorbidities). Performing investigations in experimental systems that recreate some endpoint of disease fits with the concept of drugging the diseased state. However, it would be advantageous to have preventative therapeutics to influence disease progression. It is informative to consider this process as involving multiple transitions from a normal state through intermediate stages (termed "adaptive decline"[19,35]) into an abnormal (final) state. According to this concept, the very early stages of disease are associated with changes in biological function that may not give rise to measureable alterations in a chosen model of disease. Figure 4 considers this issue by depicting the inherent disequilibrium in the observable 'steady states' as the normal scenario becomes abnormal. To illustrate this concept within the constraints of the present article, we have restricted our focus to the maladaptive 'decline' of a biological system i.e. the concept of disease progression as the manifestation of reduced complexity. This view is corroborated by models of cellular and organ dysfunction [49, 50] but we should point out that the progression of some diseases (e.g. cancer) are associated with an increased tissue complexity (if one were to take the augmented degree of tumor heterogeneity as an index of complexity), unpredictable disease behavior, and reduced efficacy of therapy [7].

Figure 5 highlights how it is possible to configure assays (including the appreciation of the importance of frequent sampling) to probe and quantify changes in dynamic experimental systems that distinguish mechanisms of drug action. Similar concepts may be applied towards understanding the increasing risk liabilities involved when considering the safety of novel drugs, which often involves multiple factors which together define overall safety. The application of gene-engineering approaches should provide the ability to induce genetically-derived disease progression models to use for assessing a drugs' effects on altered networks linked with disease progression. Perhaps in the future the idea of drugging an established diseased state

will be considered as outdated, instead being replaced by pro-active, preventative regimes, based on an improved knowledge on the dynamics of disease progression.

A note about the configuration of components within networks. The interaction of 'nodes' and 'hubs' in a network is not endlessly configurable nor is the functionality of individual components. By way of example, previous estimations have shown that, in principle, phosphorylation of a single (tetrameric) ryanodine receptor by CaMKII, if each monomer has up to eight independent CaMKII phosphorylation sites, could result in around 10^{10} different states [51]. It is not plausible that the vast majority of these configurations will exist in situ, nor that there will be a discrete functional response to the few phosphorylated configurations that will be achieved if phosphorylation represents a simple 'on-off' biological switch to regulate downstream targets. Networks constrain the function of key nodes to tolerable configurations that dictate how a particular system can behave. We consider this in more detail in section 4.

4. Defining the physiological gamut and systems limits.

In section 3, we outlined some of the issues relating to the development of assays that can resolve the dynamic nature of pathogenesis. Based on this, one might reasonably argue that we are at - or indeed, have already surpassed- the point of our ability to use simpler *in vitro* experimental systems to advance further our efforts to discover novel mechanism-based therapeutics. Maybe we are now in a phase of diminishing returns? Returning to the problems discussed in section 2 about bridging 'scale', complex interactions between the layers of genetic, epigenetic, metabolic and environmental factors cannot be replicated in constrained 'wet-lab' experiments [33]. For example, in Figure 3 we schematized a normal state in which each component in the network is well-aligned and has normal functionality, and an abnormal state which is associated with misalignment within the network space but in which the components are (in isolation) within limits of functional normality. Such a scenario could manifest as a changed 'behaviour' in a complex experimental system but elucidating this type of scenario is especially difficult. It can only conceivably be achieved using future approaches dependent on more complex human-based systems (e.g. organ-on-a-chip models), *in silico* simulations and computer modelling that integrate an array of pre-clinical and clinical data.

Contemporary *in silico* simulations represent powerful adjunctive models for drug development that are reproducible and accurate. However despite the accuracy of input parameters such models may yield outputs that may not be all that *believable* or *physiologically relevant*. To this end, an appreciation of the 'physiological gamut', that considers variability and the operable range of biological processes under normal and diseased conditions [52, 53, 54] has permeated current thinking. Most physiological processes exist with remarkably tight ranges (body temperature, pH, extracellular ion concentrations). For example, systemic central

control of human body temperature [55]. involves a number of components whose function may fluctuate substantially but the 'network' physiological response is constrained to a normal value of approximately 37.5ºC. Temperatures exceeding 40ºC are taken as a sign of high fever and those below 35 ºC hypothermic. Thus, the biologically relevant operable range of human body temperature that is compatible with a viable (i.e. living!) system is around 5ºC (± 2.5ºC; a gamut of 11% around the 'normal' value). It is therefore appropriate to question whether incorporating data acquired from experimental systems at lower temperatures (e.g. room temperature (23ºC, 32% outside of gamut) in computer simulations would provide misleading information rather than advance our mechanistic understandings [56]. Acutely aware of this problem, Windley and colleagues have recently proposed that data gathered at physiological temperatures should be used to constrain in silico models used for proarrhythmic risk prediction [57].

It is already recognized that in silico models should not operate in out-of-gamut scenarios beyond the realms of physiological believability [58]. Conversely, there are arguments that data acquired in non-human animal models do not represent the full breadth of the 'human gamut'. For example, in-breeding has produced widely-used animal models of limited genetic diversity although there is strain-to-strain variation in phenotype [59, 60] and new resources exist to reconcile background genetic variation with biologic response [61] Moreover in animals, the underlying biology may occupy a different physiological space (different networks/systems) in which the mechanisms of regulation and control are distinctly different from those in human. For example, mouse hearts beat much faster than human hearts (500-700 versus 50-70 bpm at rest), have different configuration of cellular ion pumps and exchangers for maintaining cellular ion fluxes during the cardiac cycle [62] and are resistant to ventricular fibrillation.

Approaches that incorporate the randomization of in silico parameters, non-linear modelling and chaos theory [63] together with the application of machine learning tools [64] and self-correcting parameterization [33] may lead to a better representation of 'real world' scenarios. Drug development will also benefit from input data acquired from a broader palette of studies performed in humans e.g. new approaches to map to progression of sub-clinical cardiovascular disease [65, 66]. Factors that contribute to towards variability and mosaicism in in vitro platforms are being elucidated and may also help to explain why developing the next generation of drugs requires more than an understanding of the underlying biology [35, 67, 68, 69, 70, 71, 72, 73].

Conclusions

We are some way off fully being able to annotate, interrogate and quantify some of the most interesting aspects that underpin biological complexity. Clearly, understanding the strengths and limitations of in vitro and in vivo models in terms of their complexity and ability to

recapitulate key elements of a disease phenotype is essential to developing novel pharmacological therapeutics. Werner Heisenberg is quoted as saying "….what we observe is not nature itself, but nature exposed to our method of questioning." [74]. With regards to drug discovery efforts, selecting the most appropriate experimental models (which represent our "method of questioning") and interrogating these models with the best available tools and relevant endpoints (how we expose and observe nature) is essential to discovering sorely needed novel therapeutics. There is no one-size fits all solution; consideration of relevance and level of complexity in the context of selecting different models at different phases of the drug discovery process is essential as one proceeds from drug screening to drug testing in the ultimate and most relevant of complex systems, namely patients. Complexity rules.

Figure Legends

Figure 1. Horizontal and vertical network integration in quantitative and systems pharmacology.

Horizontal integration is defined by Sorger and colleagues as the study of multiple receptors, signaling networks, metabolic pathways or cell types at the same time. Vertical integration involves linking information together at multiple spatial and temporal scales and at different levels of biological complexity. Figure is from [9] and is used under a CC-BY-SA3.0 licence. PK/PD, pharmacokinetics/pharmacodynamics.

Figure 2. Complexity beyond signaling nodes at the cellular level.

A cartoon scheme of the signaling events through which adrenaline (Adr) binding to the β-adrenergic receptor (β-AR) results in the activation of physiological "fight-or-fight" mechanisms. The depiction of the β-AR activation cascade as a simplified linear 'cause-and-effect'-type scheme though omits the complex regulation by feedback and feedforward events achieved by embedding this signaling node in a wider signaling context. Interlocking processes that underpin cellular metabolism (green) and signaling (blue) illustrate the intricate linkage of biological components [75]. A detailed map of all of the components schematized here is available at http://biochemical-pathways.com/#/map
Adr, adrenaline; AC, adenylate cyclase; Gα β γ, G-proteins; PKA, protein kinase A; reg, regulatory PKA subunit; cat, catalytic PKA subunit.

Figure 3. Hierarchical network organization and perturbation.

In this cartoon, network organization is given by the intrinsic modulation of excitation-contraction coupling (ECC), the process by which electrical excitation of cardiac cells is transduced to the physical contraction of the myocardium [62]. Ca^{2+} release from intracellular sarcoplasmic reticulum stores (SR) is regulated by layered interaction with other processes of increasing complexity (e.g. metabolism, intercellular synchronization and subcellular ultrastructure). Under normal conditions these regulated interactions are well-ordered but become disrupted in pathological states (disease) or when disrupted by toxic drugs. This scheme also conceptualizes the idea that abnormal phenotypic states (i.e. disease or drug-modified states) might be associated with the normal functional of individual processes but that they are misaligned relative to each other within the network space (or 'gamut' –see Section 4).

Figure 4. Mapping the trajectories of transitions between normal and abnormal states
(A) Here we depict the increased perturbation of a system via the transition of a normal state into an abnormal state (N and A, respectively) that is associated with an intrinsic loss of complexity. In the given scheme, progression between the start point (N) and end point (A) may occur via one of three different trajectories (orange, blue and red lines). Perturbation in all three pathways is increased between times 1 and 2 (T1 and T2) and at each sampling point the extent of perturbation for orange, blue and red pathways is the same. However the system complexity associated with each trajectory is reduced through different modalities where the rank order of complexity at both T1 and T2 is orange > blue > red.
(B) A model in which the maladaptation of a biological system occurs via sequential transitions through dysfunctional homeostatic states of reduced complexity ('pseudo-stable states') precipitated by vertical crises has been proposed.[34, 51] An expanded view of transitions through pseudo-stable states (i.e. a downward staircase) at T1 and T2 in each of the three trajectories shown in (A) is given. The vertical points of transition may correspond to points of network fragility described by Verspignani [42]. The blue pathway is characterised by homogenous transitions between pseudo-stable states over the entire N-to-A transition. In contrast, the orange and red pathways proceed via heterogeneous transitions to reduced complexity states.

Figure 5. Exploring complexity in a model of human cardiac cell signalling
We illustrate the concepts described in Figure 4 using signals representing spontaneous Ca^{2+} oscillations in human cardiomyocytes exposed to drugs A and B and sampled at 5 times points (**1**-**5**). Drugs A and B both elicit irregular beating and eventual cessation of calcium oscillations along with reductions in signal amplitude. Sampling only at time point **5**, it would be correct to conclude that drugs A and B produce the same phenotypic outcome (i.e. elimination of signal spikes). However, more regular sampling would reveal that cells treated with drugs A and B exhibited comparable oscillatory behaviour at sampling times **1** and **2,** which then had diverged by **3** and **4** only to re-converge on a common endpoint at **5**. Drugs A and B thus result in comparable outcomes (catastrophic perturbations in signalling) via different trajectories. In (B), the differences in the signalling patterns of Ca^{2+} oscillation evoked by drugs A and B can be resolved using new methods for decoding cell signalling information [76, 77].

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Expert opinion (997 words).

Calls for improved target-based screening and phenotypic screening to improve drug discovery and development are not new, nor are calls for more input from systems biology to assist in understanding the key elements in complexity that might be considered as mechanistic-based disease targets in the future. The enabling technologies are becoming more sophisticated, but we must ask new questions of them whilst recognising their present limitations in a way that moves drug development forward. Each approach must be applied at the proper time in the discovery/development pipeline (and with appropriate data) in order to provide holistic pre-clinical assessments of drug safety and efficacy. Contextualization is key. For example, a generalized approximation of an organ's 'physiologic state' may be inferred from functional readouts, detailed information relating to cellular ultrastructural organization and tissue architecture and rich 'omics'-level data (e.g. transcriptomic, genomic, proteomic and metabolomics). However, at present, no single experimental model can provide these data in the correct context, at all levels of resolution and across all scales of complexity. It would be expected though that in any chosen model system relevant component nodes and networks should exist (e.g. syncytial networks of stem-cell derived cardiomyocytes in vitro should possess functional β-AR signalling machinery (Figure 2), despite the (typical) lack of sympathetic innervation in culture). Whether all of this information that emerges from different experimental models can be integrated holistically through 'network inference'- based on the widely-held view that biological processes exist exclusively in 'scale-free' or 'heavy-tailed' networks consisting of multiple interconnected nodes- has been challenged by evidence that scale-free networks may not be all that prevalent in real-world scenarios [78, 79].

We have presented evidence that biological processes are complex on multiple horizontal and vertical levels of organization, and that popular target-centric approaches used in drug discovery efforts are incomplete in many aspects. In addition, our understanding of even the simpler (cellular) levels of complexity is also often incomplete and inadequate. Issues related to low throughput, and difficulties in properly interpreting results from more complex models hinders their use in early drug screening and efforts to replace simpler target-centeric screening. Thus, despite continuing technological innovation, drug discovery efforts remain exceedingly difficult, time-consuming, and expensive. In this article we have made the case that comprehensive drug screening requires a multi-scale approach encompassing all levels of complexity from reductionist investigations on single molecules to unequivocally resolve drug-target interaction through to systems-level studies to interrogate 'big data' from populations of patients. We recognize that multi-faceted data almost always allows questions to be asked which the originators of the component datasets did not think to ask, perhaps because the requisite contextualisation (the "big picture") was missing or that the full value of combining the

data with other datasets was not realised. Thus sharing good quality, structured datasets in searchable archives will be vital in allowing the events underpinning more complex biological scenarios to be properly synthesized.

None of this is all that disruptive; we have presented some actionable items which we consider to be a directed evolution rather than revolution. A future challenge to drug discovery efforts is how best to blend the best output of systems biology approaches and experimental humanized models to guide discovery efforts. This will entail greater communication between more traditional "wet lab" and "computational biologists" and informatics to enable/gain a better understanding of drug effects on normal and disease states. From the present vantage point though, enthusiasm toward the improved power to crunch huge numbers should be tempered by an awareness that the outcomes may not be all that useful if we do not understand fundamental characteristics of the platforms that give rise to the numbers in the first place ("BIG DATA, little understanding). We are optimistic that it is a matter of time until drug development emerges from a hinterland where "data rich" is often not very helpful, "stem cell" preparations are still evolving, and an appreciation of biology from a "network" perspective is still not very mainstream. In order to help connect the data emerging from these evolving experimental models with computational modelling and forge new paths forward in understanding how complexity affects drug responses, the drug discovery process (and eventually, efficacy) will rely on enhanced use of informatics and applied statistics (e.g. mediation analysis [80]) which are absolutely necessary to untangle issues on inference and causality.

While efforts to adapt model systems continues, the pace of evolution though is probably too slow for some and drug hunters cannot wait. Humanized integrated systems (in the form of human stem-cell derived preparations) offer an alternative to animal models with the potential benefit of providing more complex integrated systems that closely resembling human biology. More recent work with such preparations have shown promise in modelling patient-specific diseases, with the cells (or engineered tissues) providing integration of signals. The extent to which such humanized systems ("proclinical studies") replicate native responses will need to be constantly evaluated as these models evolve in engineered (and biological) complexity. It is worth emphasizing that by definition, no model is perfect, but some may be "fit for purpose". We would offer a note of caution also that the interpretation of outputs from humanized models needs to separate the 'usability' of the model from its usefulness. To this end, it is important that we make every effort to record and annotate as many aspects of the biology and phenotype as possible- especially those parameters that are more difficult to quantify but may unmask a new level of complexity and eventual understanding (e.g. the visible granularity of nuclei following exposure to drug). We need to pre-empt retrospective mutterings of "if only we'd thought to record that at the time" wherever possible even though reconciling observation with underlying mechanism(s) remains a considerable challenge.

Such demands will require a different type of scientific training and collaboration than is not demanded by typical reductionist approaches. Perhaps a joint industry/governmental group could take up the challenge to guide such an educational initiative. The industry, government regulators, and patients are waiting.

Article highlights (182 words)

- A lack of understanding of the complexity of biology in health and disease remains a key issue in limiting the discovery of novel pharmacologic therapeutics.
- Systems-biology approaches and computational models provide alternative frameworks to test drugs (eventually beyond animal models).
- Human stem cell-derived preparations represent an evolving experimental approach to interrogating drug efficacy and drug safety in the proclinical space (preclinical studies using human-derived “clinical-like” cells or tissues). Further work is ongoing to define the minimum systems necessary to recapitulate the critical processes in healthy and diseased states (for safety and efficacy studies, respectively).
- Experimental systems need to be configured and sampled in ways such that data output reflects the dynamic (temporal) changes underpinning disease-linked or drug-toxicity evoked phenotypes.
- The integration of multiple network- and systems-based responses should provide better and more comprehensive assessments of drug effects compared to more traditional target-centric approaches to drug discovery.
- Phenotypic screening using human stem cell-derived cells and tissues represent a complementary approach to quantitative systems biology-based studies that include higher levels of integration and complexity for evaluating drug candidates.

Figure 1

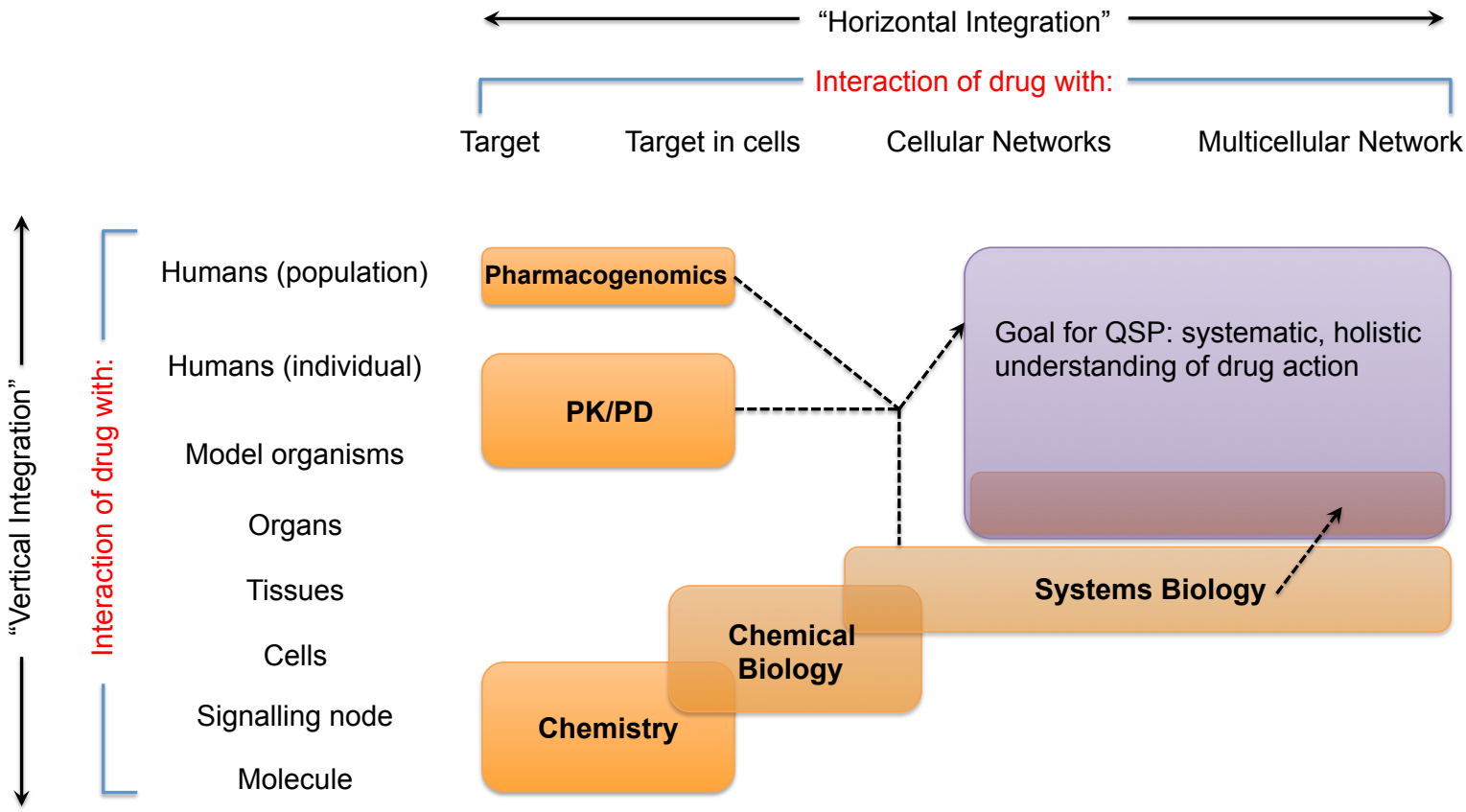
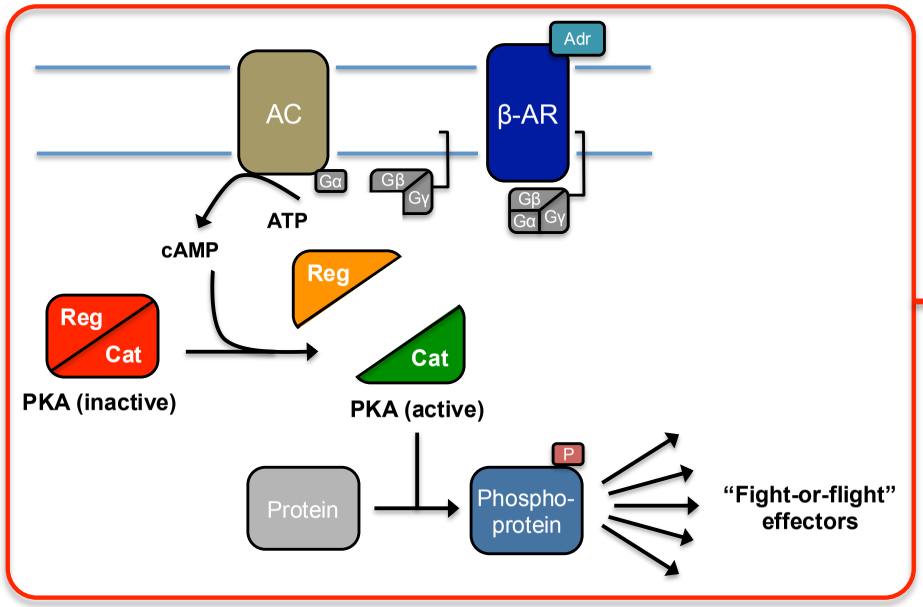

"Horizontal Integration"
Interaction of drug with:
Target
Target in cells
Cellular Networks
Multicellular Network
"Vertical Integration"
Interaction of drug with:
Humans (population)
Humans (individual)
Model organisms
Organs
Tissues
Cells
Signalling node
Molecule
Pharmacogenomics
PK/PD
Chemistry
Chemical Biology
Systems Biology
Goal for QSP: systematic, holistic understanding of drug action

Figure 2



Purines
NAD/NADP
Acidic carbohydrate derivatives
Histidine
Ino-sitol
Pentose cycle
Lysine
Di- / poly-saccharides
Amino sugar derivatives
Serine, threonine, cysteine and methionine
Nucleo-tide sugars
Pyruvate
Citrate and glyoxalate cycle
Porphyrins
Heme and cytochromes
Glycolysis and gluconeogenesis
C1
Glyco- and phospholipids
Fatty acids
Urea cycle
Glutamate, proline and hydroxyproline
Pyrimidines
Sphingolipids
Leucine, isoleucine and valine
Carotenoids and isoprenoids
Mineralocorticoids and glucocorticoids
Androgens and estrogens
Coenzyme-A
Sterols

Immune system
Blood coagulation
Ion channels
Plasma membrane receptors
Translation
Post-translational modification
IP_3-regulation
Ca^{2+}-regulation
Gly-cogen
Transcription
Eicosanoids
Steroid hormones
DNA replication

Figure 3

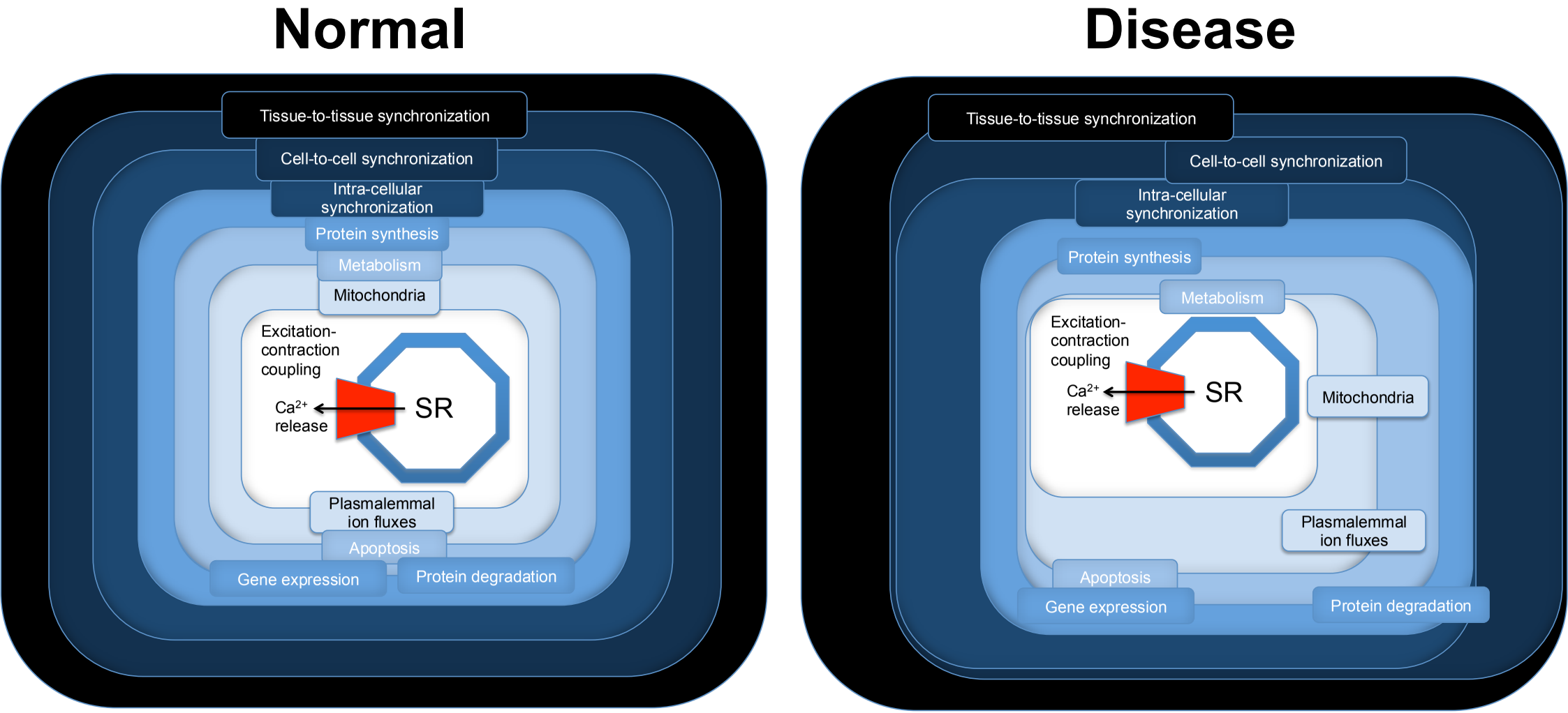

Normal
Tissue-to-tissue synchronization
Cell-to-cell synchronization
Intra-cellular synchronization
Protein synthesis
Metabolism
Mitochondria
Excitation-contraction coupling
Ca2+ release
SR
Plasmalemmal ion fluxes
Apoptosis
Gene expression
Protein degradation
Disease
Tissue-to-tissue synchronization
Cell-to-cell synchronization
Intra-cellular synchronization
Protein synthesis
Metabolism
Excitation-contraction coupling
Ca2+ release
SR
Mitochondria
Plasmalemmal ion fluxes
Apoptosis
Gene expression
Protein degradation

Figure 4

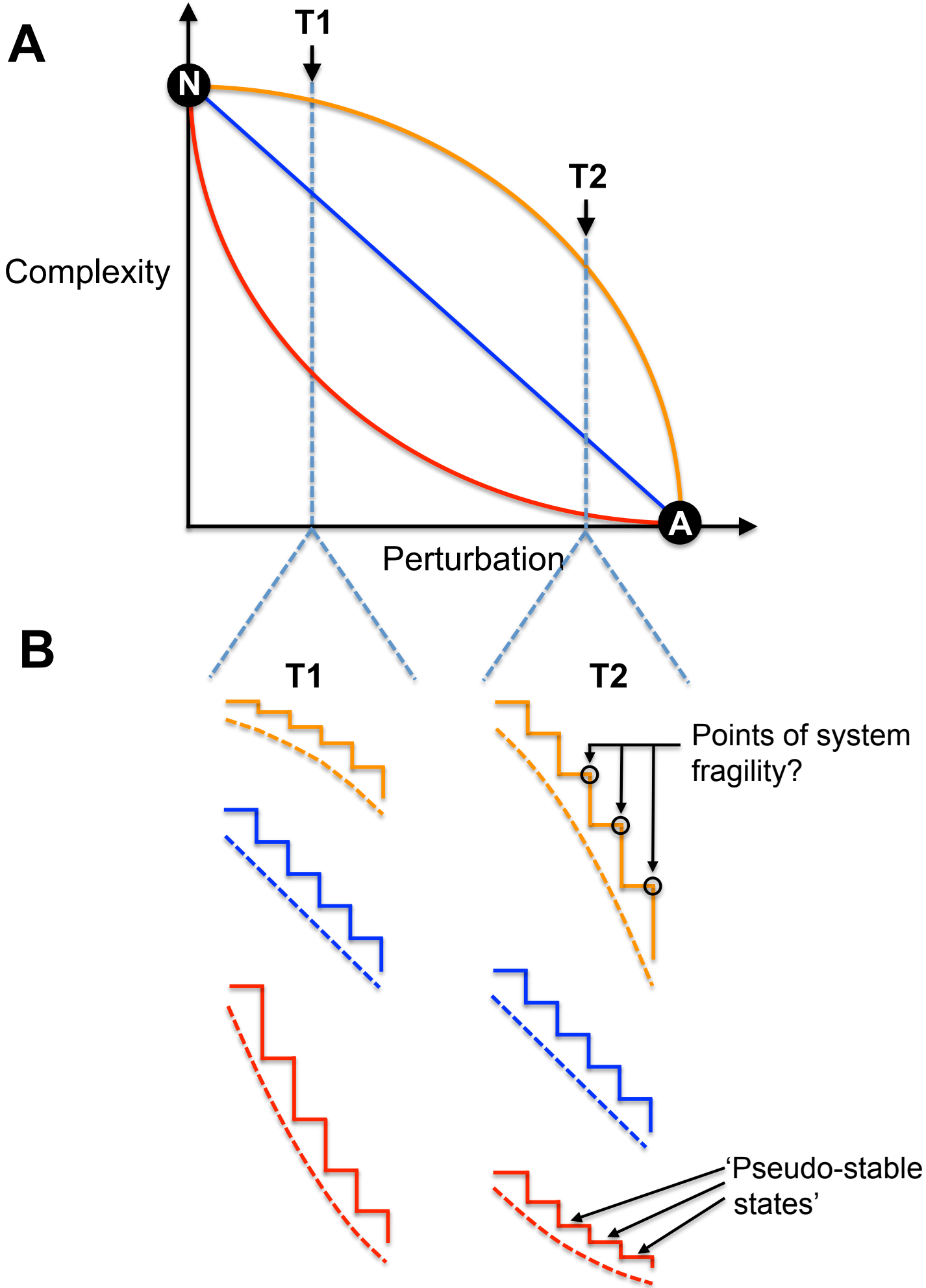
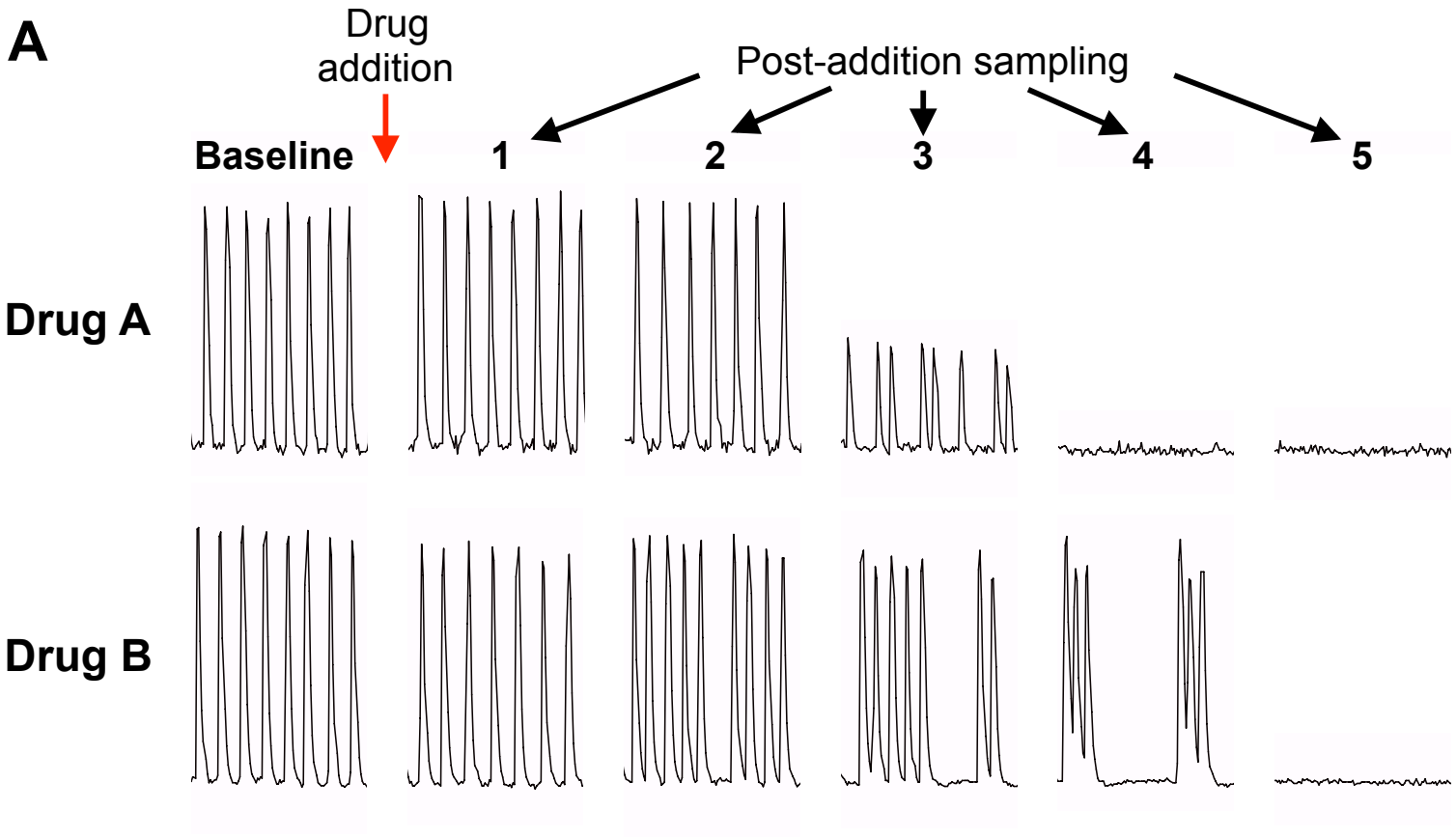
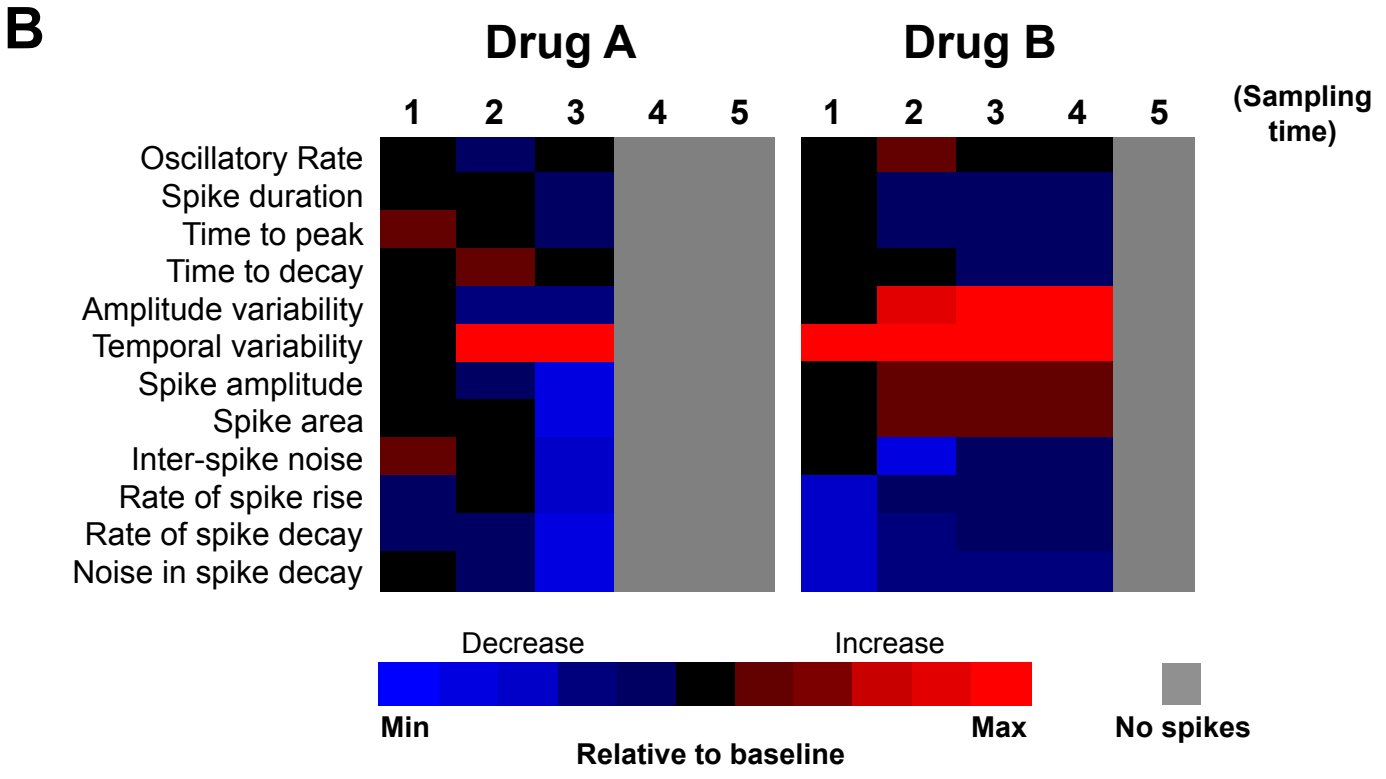

A
T1
T2
N
A
Complexity
Perturbation
B
T1
T2
Points of system fragility?
'Pseudo-stable states'

Figure 5


A
Drug addition
Post-addition sampling
Baseline
1
2
3
4
5
Drug A
Drug B


B
Drug A
Drug B
1 2 3 4 5
1 2 3 4 5
(Sampling time)
Oscillatory Rate
Spike duration
Time to peak
Time to decay
Amplitude variability
Temporal variability
Spike amplitude
Spike area
Inter-spike noise
Rate of spike rise
Rate of spike decay
Noise in spike decay
Decrease
Increase
Min
Max
No spikes
Relative to baseline