Integrative and Comparative Biology

Integrative and Comparative Biology, volume 57, number 2, pp. 171–184
doi:10.1093/icb/icx076

Society for Integrative and Comparative Biology

INVITED REVIEW

Systems Biology of Phenotypic Robustness and Plasticity

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Synopsis Gene regulatory networks, cellular biochemistry, tissue function, and whole body physiology are imbued with myriad overlapping and interacting homeostatic mechanisms that ensure that many phenotypes are robust to genetic and environmental variation. Animals also often have plastic responses to environmental variables, which means that many different phenotypes can correspond to a single genotype. Since natural selection acts on phenotypes, this raises the question of how selection can act on the genome if genotypes are decoupled from phenotypes by robustness and plasticity mechanisms. The answer can be found in the systems biology of the homeostatic mechanisms themselves. First, all such mechanisms operate over a limited range and outside that range the controlled variable changes rapidly allowing natural selection to act. Second, mutations and environmental stressors can disrupt homeostatic mechanisms, exposing cryptic genetic variation and allowing natural selection to act. We illustrate these ideas by examining the systems biology of four specific examples. We show how it is possible to analyze and visualize the roles of specific genes and specific polymorphisms in robustness in the context of large and realistic nonlinear systems. We also describe a new method, system population models, that allows one to connect causal dynamics to the variable outcomes that one sees in biological populations with large variation.

Introduction: robustness and plasticity

Animals are dynamically changing systems that operate in complex and variable environments. They are composed of diverse physiological and biochemical systems that provide a division of labor and compartmentation of various specialized function. All these functions need to be integrated to allow the animal to respond to daily and seasonally changing variables in its environment, such as nutrition, temperature, water availability, toxins in food (e.g., plant secondary compounds), and pathogens that populations experience throughout their existence. In addition, these functions and interactions must be designed so that they operate without fail even in the face of a large and diverse amount of individual genetic variation. Biological systems have evolved a variety of homeostatic mechanisms that stabilize the form and function of their phenotypes against genetic and environmental variation. Indeed, these mechanisms have evolved at all levels of the organizational hierarchy from gene regulatory and metabolic systems that operate in cells tissues and organs, to physiological systems that operate across the entire body. Collectively, these stabilizing mechanisms convey phenotypic "robustness" so that animals continue to operate reasonably normally in the face of often exceptionally challenging genetic and environmental conditions.

The concept of robustness was first developed by Waddington (1942). It arose from a series of observations about genetics and development. Genetic studies showed, for instance, that mutants were almost always more variable than wild types, and early studies in development had shown that when a piece of a tissue or organ is removed early in development subsequent growth compensated and resulted in a final structure without defect. Waddington postulated that mechanisms have evolved that stabilize the phenotype against both genetic and environmental perturbation. He called this "canalization" and contrasted that with developmental "flexibility" in

Advance Access publication July 14, 2017

which a phenotype changes, adaptively, with a change in environment (Waddington 1957). Today we call these phenomena robustness and adaptive plasticity, respectively.

There has been a long-standing interest in understanding the evolutionary causes and consequences of robustness and plasticity, and also in understanding the genetic and developmental mechanisms that make phenotypes plastic, or make them insensitive to genetic and environmental variation (Wagner 1996; Felix and Barkoulas 2015). Robustness and plasticity pose interesting evolutionary problems because both decouple genetic variation from phenotypic variation. Insofar as selection acts on phenotypes, while only genes are transmitted from one generation to another, if there is no correlation between genes and traits it was difficult to see how robustness and plasticity could evolve.

Substantial theoretical work has been done to show the conditions under which robustness and plasticity can evolve. Much of this work is based on statistical genetics (Via and Lande 1985; Wagner et al. 1997; Rice 1998; Gibson and Wagner 2000; Kawecki 2000; Lande 2009), or uses simulations with small networks (Wagner 1996; Frank 1999; Becskei and Serrano 2000; Omholt et al. 2000; Gibson 2002; Meir et al. 2002; Flatt 2005; Ciliberti et al. 2007) to deduce general conditions, like network topology, degree of modularity, nonlinear interaction, fluctuating environment, and variance–covariance structure, under which selection could lead to phenotypic stability or plasticity.

Mechanisms of robustness

Most research that has explicitly addressed mechanisms and the systems biology of robustness and plasticity has been done using small networks, or larger networks with simple Boolean or linear mass-action kinetics (Bolouri and Davidson 2003). Relatively little work has been done with large systems using the real kinetics by which the component parts interact.

There are several compelling reasons why many system biologists have stayed away from working with real biological systems: biological systems are incredibly complex; most interactions within those systems are non-linear; the structure or topology of the systems are not fully known; the kinetics by which the parts interact are not fully known; systems are not static but dynamic and change over many time scales; and, finally, interactions occur simultaneously between many levels of the biological hierarchy from molecules to cells to tissues to organisms. All of these features, and deficiencies in our knowledge, make understanding how these systems operate, how they evolve, and how they acquire robustness, exceptionally challenging.

The general mechanisms by which real systems obtain robustness are understood, at least in principle. Felix and Wagner (2006) distinguish between distributed robustness and redundancy in signaling and metabolic pathways. In distributed robustness there are many pathways from A to B, each with different properties and kinetics, and in redundancy there are many identical parallel pathways between A and B. Wagner (2000) earlier showed that in yeast it was likely that robustness was primarily due to reactions among unrelated genes and not due to duplicated genes, suggesting that robustness is a distributed process and probably evolved in response to stabilizing selection.

The actual mechanisms that provide robustness are numerous and diverse. Feedback and feed forward mechanisms in physiology and biochemistry are probably the best known (Alon 2007). Barkai and Leibler (1997) showed how feedback in simple metabolic networks can lead to robustness of enzyme activity around a setpoint, and that the setpoint can be altered by changing the parameters of the system. Parallel pathways are thought to convey robustness in gene regulatory and biochemical networks (Wagner 2000), as are substrate inhibition mechanisms (Reed et al. 2010). In development, lateral inhibition and Turing-style reaction-diffusion mechanisms (Turing 1952; Meinhardt 1982; Meir et al. 2002; De Joussineau et al. 2003; Salazar-Ciudad et al. 2003) with short range autocatalysis and long range inhibition produce bistable spatial patterns of high and low activator biosynthesis. The patterns produced by reaction-diffusion mechanisms are, however, very sensitive to parameter values and to the dimensions of the field (Murray 1982), so their robustness is highly conditional. Diffusion gradient-threshold mechanisms, with a morphogen gradient between a source and a sink, can produce stable and robust patterns that are insensitive to the dimensions of the field (Wolpert 1969, 1994). The *Drosophila* segmentation and segment polarity systems are made up of a sequence of diffusion-gradient-threshold steps which have been shown to produce a robust spatial patterns (von Dassow et al. 2000; Houchmandzadeh et al. 2002; Eldar et al. 2004).

The bistable switch-like behavior of signaling cascades (Ferrell 1996; Huang and Ferrell 1996) is a form of robustness, where the system is insensitive to variation in input at low input levels, followed by a switch to high activity over a small range of intermediate inputs, and stable again to variation in input at high input levels. This kind of robustness emerges simply from the kinetics of the mechanism and is

characteristic of multi-step cascades (Ferrell 1996). Dominance is also a kind of robustness in which heterozygotes with only half the gene dosage, nevertheless, have a phenotype identical to one of the homozygotes. Kacser and Burns (1981) showed that dominance can be an emergent property of a system with sequential enzymatic steps. Flux through such a reaction chain becomes an increasingly non-linear function of enzyme activity with increasing chain length. Non-linear mechanisms also operate throughout physiology and development (Omholt et al. 2000; Gilchrist and Nijhout 2001), so dominance as a robustness mechanism is a systems property that is not limited to biochemical systems but widespread, operating at all levels of the organizational hierarchy.

Most systems probably have multiple robustness mechanisms operating to stabilize different aspects of the phenotype against different kinds of genetic and environmental perturbations. de Visser et al. (2003) describe three general evolutionary causes of genetic robustness: adaptation, when the robust phenotype is more fit than the alternative; intrinsic, when robustness is an inevitable consequence of a particular biochemical or developmental mechanism; and congruent, when it is a consequence of the evolution of environmental robustness. Reaction-diffusion, gradient-threshold, and the various non-linear processes, outlined above, that produce robustness, switch-like behavior, and dominance, may be examples of intrinsic causes of robustness, although it would be hard to prove that they did not evolve gradualistically, via adaptation. Some of the systems we will outline below have robustness to both environmental and genetic perturbation and may be examples of congruence.

Although selection favors phenotypes that are robust to genetic and environmental variation it must also favor novel phenotypes that permit adaptation to a changing or new environment. Phenotypes are therefore said to "walk a tightrope" between the apparently contradictory requirements of stability and change (Padilla and Tsukimura 2014; Padilla et al. 2014). One form of flexibility is phenotypic plasticity in which a phenotype changes when the environment changes. The norm of reaction, which is a systematic change in phenotype along an environmental gradient (Schlichting and Pigliucci 1998), is an example of phenotypic plasticity, as is polyphenism, the development of alternative phenotypes in response to environmental signals (Pfennig 1992; Nijhout 1999, 2003; Abouheif and Wray 2002; Moczek and Nijhout 2003; Simpson and Sword 2009).

Phenotypic stability in form and function is not due to a fixed steady-state but is a dynamic systems property that is actively maintained by opposing mechanisms. For example, in many tissues there is continual cell death and replacement, and this structural turnover occurs without change in morphology or function. And in physiological systems stability is maintained by the balance of competing mechanisms. Homeostatic mechanisms also stabilize many biochemical and metabolic functions in cells and organs against variation in input and demand. All homeostatic mechanisms operate successfully only over a finite range of input values as illustrated in Fig. 1. Within the homeostatic range of the genetic variable, the phenotypic variable varies very little, but outside that range the phenotypic variable changes rapidly fueling natural selection with new variation. We call this "escape from homeostasis" and give many examples in Nijhout et al. (2014). As we will see in Example 1, a change in environment can also tip a flat homeostatic curve, and this also allows natural selection to act.

Mathematical models of biological systems

Understanding homeostatic mechanisms, how they are created and the ranges over which they operate, is fundamental to understanding robustness in biological systems. The properties of some regulatory mechanisms, for example feedforward or feedback control, are well-understood in isolation (Mangan and Alon 2003; Alon 2007), but in reality they operate within large networks with many such overlapping mechanisms. To study such large, non-linear systems, and understand their properties, it is essential to build mathematical models. Complex non-linear systems simply cannot be understood by intuition.

There is a long tradition in physiology and biochemistry of building ordinary differential equation (ODE) models of a broad diversity of systems (Guyton et al. 1972; Ten Tusscher et al. 2004; Keener and Sneyd 2009). One of the challenges in building a model that accurately simulates the properties of a biological system is that we often do not know the entire network that might be relevant, nor all the kinetics that link all the variables. So how does one determine whether a model of a given system is any good? The standard test is to determine if the model accurately simulates data that were not used in building the model in the first place. For instance, does the model do both time-evolution after a perturbation, and dose–responses to varying inputs, correctly without having to fiddle with the parameters? Can the model predict the outcome of a new experiment? Does the model help resolve contradictory experimental results in the literature? As a model passes successive validation tests of this

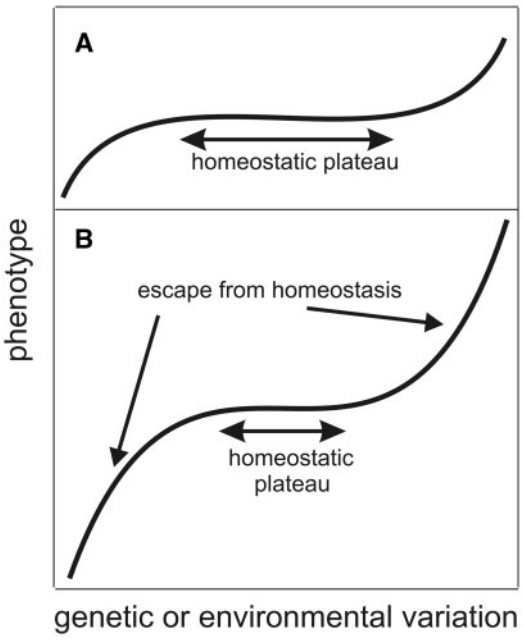

Fig. 1 Homeostasis is revealed by chair-shaped graphs between environment or genotype and a phenotype. (**A**) The homeostatic plateau is the region over which active mechanisms are able to stabilize the phenotype. (**B**) Mutations can destabilize homeostasis and reduce the width of the homeostatic plateau. This allows phenotypes to become responsive to environmental or genetic variation, a phenomenon called "escape from homeostasis."

kind one's confidence that it might be a reasonable representation of a system increases.

The ultimate aim of a model should be to help us obtain a deeper insight into how a system works. Biological networks' functions and models should help us to understand the biology. If we are to understand, mechanistically, the causes and consequences of robustness in a complex non-linear biological system we need a good mathematical representation of that system. There are relatively few systems in which this can be done, largely because of a lack of diverse and accurate quantitative data. Physiological systems and, in particular, biochemical systems, are the exceptions, because those disciplines have traditionally been the most quantitative. Biochemical systems, and some physiological systems, have the added advantage that they allow us to directly bring genes into the model. This is because enzymes, transporters, and members of signaling pathways are gene products and their activity, expression level, and effect of mutations, can be empirically measured and become explicit parts of the model.

To overcome the lack of empirical and quantitative data we study human health-related metabolic systems, because a vast and unparalleled amount of experimental data is available on network structure, enzyme kinetics, and the specific effects of mutations. Metabolic networks can be quite complicated topologically. Many are not nice linear or branched pathways but cycles, and sometimes cycles within cycles. A large number of enzymes are activated or inhibited by metabolites from elsewhere in the network. A surprisingly large number of enzymes exhibit substrate inhibition (Reed et al. 2010), which adds to the nonlinearity. Transporters import and recycle metabolites within and between cells, and the circulatory system shuttles metabolites between tissues and organs. We believe that our approach can serve as an exemplar for the study of robustness and homeostatic mechanisms in other biological systems (e.g., developmental biology) that involve genes, gene products, signaling cascades, and environmental inputs.

Individual variation and system population models

Once one has determined the coefficients in an ODE model, the steady state is determined, as is the response to external influences. Such models implicitly assume that they represent an "average" individual or an "average" cell. But cells and individuals show enormous biological variation in phenotype and it is exactly this variation that allows natural selection to operate. We introduced a method that we called, "system population models," for including biological diversity in ODE models in Duncan et al. (2013). Our ODE models contain many parameters, for example V_{max} values of enzymes that are proportional to gene expression levels. All parameters have a "normal" value for the "average" individual. We choose a new value for each parameter by selecting from a probability distribution with average value equal to the mean. We then run the model to steady state and record the parameter values, and all the concentrations and velocities. That is one virtual individual. If we do this procedure a thousand times, we then have a database of virtual individuals and we can use the usual techniques of statistical analysis to investigate the behavior of populations of individuals that have the same structure of network topology and dynamics but different parameters. Since the population of virtual individuals is based on an underlying deterministic model, when we find an interesting statistical phenomenon, we can use the underlying ODE model to determine the mechanistic reason for the phenomenon. Other uses for system population models of deterministic systems have been described by Wagner (2015).

To illustrate why and how phenotypic robustness and plasticity emerge naturally from homeostatic

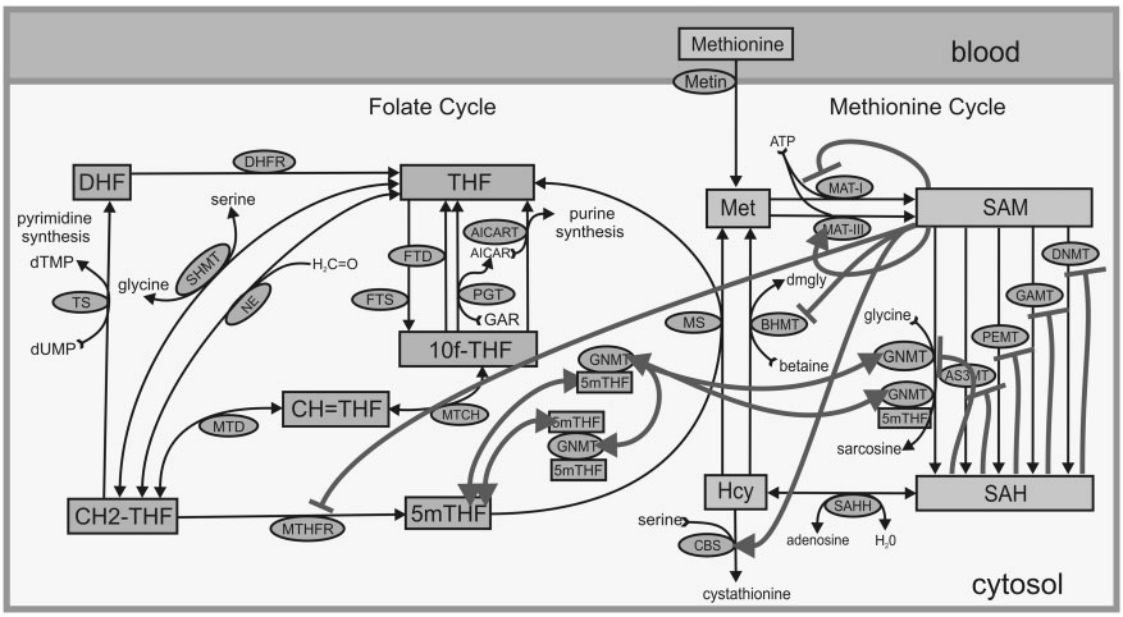

Fig. 2 FMOC metabolism. Boxes indicate metabolites and ellipses are enzymes. Narrow black arrows indicate biochemical reactions and the broad gray arrows indicate allosteric activation or inhibition or dimerization reactions. Names of acronyms used in this paper are in the text, others can be found in Reed et al. (2008). Data on the structure of the mathematical model can also be found in Reed et al. (2008).

systems, we discuss four specific examples where the application of ODE population model approaches provides insights into how system robustness is achieved from plasticity in the underlying components (e.g., enzyme activity) of biological systems. We will discuss four examples of system robustness and the use of system population models. Below, we discuss robustness and cryptic genetic variation in models of folate-mediated one-carbon (FMOC) metabolism. We show how system population models can give real information about the underlying physiology in FMOC. We then show the use of system population models to understand the meaning of "predisposition" to disease using a model for dopamine metabolism. Finally we use a model of serotonin metabolism to show how system population models allow one to identify subpopulations that have efficacious or deleterious reactions to drugs. We conclude by showing why and how phenotypic plasticity emerges naturally from homeostatic systems.

Robustness and cryptic genetic variation

FMOC metabolism is a complex metabolic network made up of the folate cycle and the methionine cycle and glutathione biosynthesis. Figure 2 illustrates a portion of this reaction network and is shown here primarily to illustrate the complexity of the topology: the network is made up of several interlocking cycles so that every enzyme and metabolite is both upstream and downstream of all the others. FMOC derives its name from the fact that it takes in several amino acids and strips off one-carbons as methyl groups that are carried by folate derivatives and are then used in biosynthesis. For instance the AICART and TS reactions are the early steps in the de novo biosynthesis of nucleotides, and the TS reaction is the rate limiting step for DNA synthesis. The system also contains the DNA methylation reaction (DNMT) and is therefore involved in epigenetic modification. Defects in one carbon metabolism are associated with birth defects like spina bifida, various cancers especially colorectal cancer, cardiovascular disease, and several psychiatric disorders. Our mathematical models for FMOC have revealed multiple regulatory interactions (shown by the thick gray arrows in Fig. 2), in which metabolites activate or inhibit enzymes in the network. Our analysis has shown that these regulatory interactions serve to stabilize a few specific reactions in the system, namely: the TS reaction which is the rate-limiting step for DNA synthesis, the AICART reaction which is an early step in the de novo synthesis of nucleotides, the DNMT reaction which ensures correct methylation of newly replicated DNA, and also the synthesis of glutathione (not shown) (Nijhout et al. 2004, 2006b; Reed et al. 2006, 2008). As amino acid input into the system varies, for instance with meals, these homeostatic mechanisms maintain stability of these critical reactions. Thus robustness to environmental

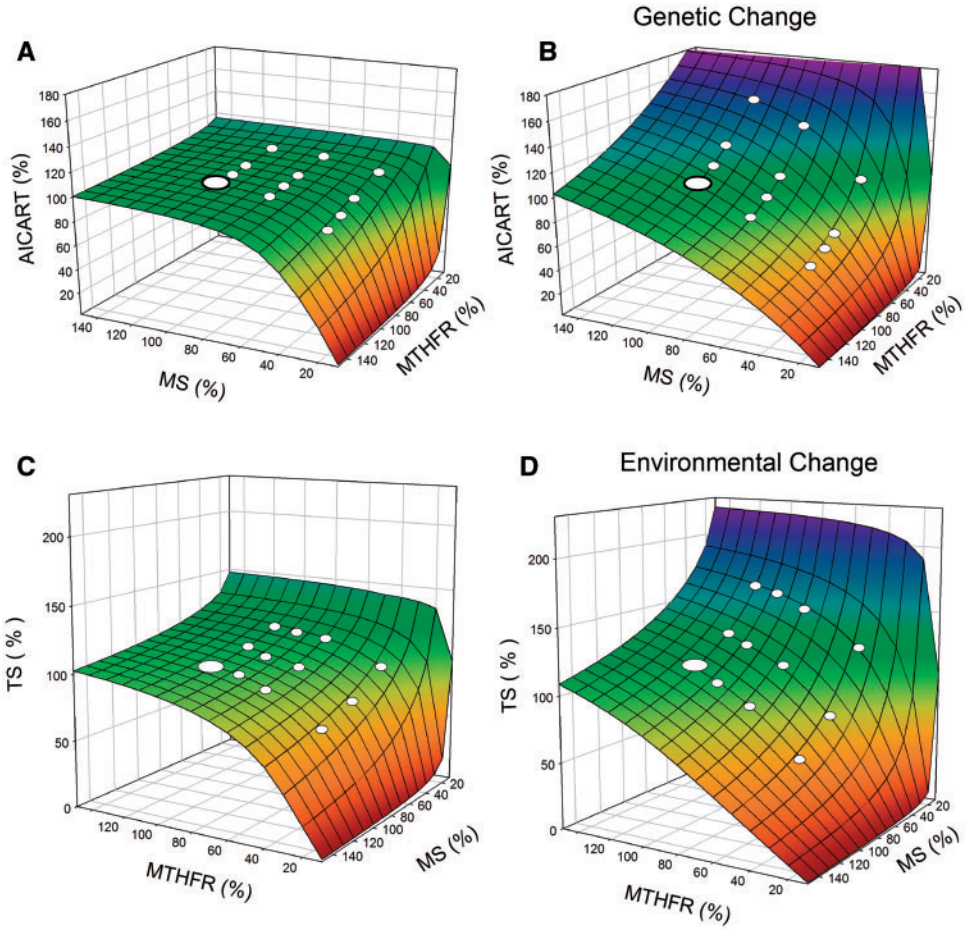

Fig. 3 (**A**) Phenotypic landscape showing the dependence of the AICART reaction on genetic variation in MS and MTHFR. The position of the wild-type genotype is shown by the largest dot, and the positions of several common polymorphisms are shown by the smaller white dots. All activities are scaled with respect to wild-type (=100%). (**B**) The same landscape after inactivation of one of the regulatory feedbacks is shown in Fig. 2. The landscape is tilted and the same polymorphisms now have a large phenotypic effect. (**C**) Phenotypic landscape showing the dependence of the thymidylate synthase (TS) reaction on genetic variation in MS and MTHFR showing the positions of the same polymorphisms as in A and B. (**D**) The same landscape in the presence of a vitamin B_{12} deficiency. The landscape is tilted and the same polymorphisms now have a large phenotypic effect.

perturbations is conveyed by the system to a few critical components, and this robustness is dynamically maintained, much in the way body temperature is maintained by continual dynamic switching between heating and cooling systems.

It turns out that these regulatory interactions also make the same four critical reactions robust against genetic variation. We think of these as the phenotypes of the FMOC system: the main outputs of the system. This is illustrated in Fig. 3A, B for the AICART reaction. This figure plots the rate of the AICART reaction (the phenotype) as a function of variation in the activities of two enzymes: methionine synthase (MS) and methylenetetrahydrofolate reductase (MTHFR). The largest spot marks the location of the normal, wild-type activity of these two enzymes. It has been surprising to find that in human populations there are high-frequency big-effect polymorphisms in genes for many of the enzymes in FMOC (for tables and additional figures, see Nijhout et al. [2015] and Nijhout and Reed [2014]), including for MS and MTHFR. These mutations drastically lower the activities of the gene products. It is notable that although the mutations have large functional consequences, for instance, reducing the activities of the enzymes to as little as 30–40% of normal, nevertheless the rate of the DNMT reaction does not vary very much. The phenotypic surface is very nonlinear and both the wild-type and the mutations lie on a relatively flat region of the surface.

The fact that the flat region of the phenotypic landscape in Fig. 3A is orthogonal to the *z*-axis is an indication of robustness to genetic variation, because genotypes on that plane have nearly the same phenotypic value. The robustness mechanism evolved such that the wild-type genotype is located

near the center of the horizontal plane, and this is why mutational variation around the wild-type has little effect on the phenotype. These homeostatic mechanisms thus allow for the accumulation of cryptic genetic variation: variation that, although it may be large at the functional level, is slight at the phenotypic level.

The idea of cryptic genetic variation was originally suggested by Waddington (1942) who noted that mechanism of canalization could mask the effects of mutations and thus allow for the accumulation of genetic variation. Canalization, rather than limiting evolution, could provide the substrate for future evolution, what today we call evolvability (Waddington 1942, 1957; Wagner 2005, 2008). Genetic assimilation and genetic accommodation both rely on the presence of hidden genetic variation that can be exposed by shifts in the genetic or environmental factors that stabilize the phenotype (Waddington 1942, 1953; de Visser et al. 2003). The relationships between robustness, cryptic genetic variation, and evolvability have been extensively studied and commented upon at the theoretical level (Newman and Müller 2000; Siegal and Bergman 2002; Gibson and Dworkin 2004; Flatt 2005; Gibson and Reed 2008; Le Rouzic and Carlborg 2008; Schlichting 2008; Wagner 2008; Kaneko 2009; Masel and Siegal 2009; Mcguigan and Sgrò 2009). Using well-validated models of metabolic systems it is actually possible to empirically identify specific genes whose effect is masked, and reveal the actual mechanisms by which robustness is achieved.

The effects of the robustness mechanism can be visualized by means of the phenotypic landscape. A phenotypic landscape is a graph of the phenotype as a function of the activities of genes products. A phenotypic landscape is a genotype–phenotype map. The shape of a phenotypic landscape is a systems property: it depends on all the variables and parameters of the system. In other words, there is no unique association between the activity of a gene and the value of a phenotype.

In the model we can introduce an environmental shift in the form of a vitamin B_{12} deficiency, which is a required cofactor for the enzyme MS. The result is shown in Fig. 3C for normal level of B_{12} and Fig. 3D for a reduced level of B_{12}. Under a vitamin deficiency the phenotypic landscape is tilted, and the mutations that formerly had little phenotypic effect now have a large one, and cryptic genetic variation is now revealed. Reducing the activity of MS could also occur through an additional mutation in the coding region of the gene, or a mutation that reduces the expression of the gene product. Thus genetic variation by mutation, and environmental variation by a vitamin deficiency have similar effects on the phenotype. The effect of environmental variation on a trait is generally considered to represent phenotypic plasticity, so we have a trait here that is robust to one kind of environmental variation (amino acid input) but not to another (vitamin B_{12} status).

Alternative stable systems

Many physiological and developmental systems have more than one stable phenotype. In development we have polyphenisms, such as the castes of social insects and the alternative seasonal forms of many insects that are controlled by shifts in hormone secretion that direct development along alternative pathways (Nijhout 2003). In reaction-diffusion systems in pattern formation there are typically two alternative steady states (Meinhardt 1982). In physiology, fever is due to a resetting of the temperature homeostatic setpoint so body temperature is now stably regulated at a higher level. In FOCM we found an interesting switch. Two enzymes in the mitochondrial folate cycle are active during fetal development but are inactive in adults, and re-activated in many cancers. In the model, when we switch between the alternative activity states of the enzymes there is an alteration of flux in the pathway so that when the enzymes are active flux favors nucleotide synthesis and when they are inactive flux is redirected to favor supplying energy metabolism (Nijhout et al. 2006a). This makes functional sense in that the enzymes are active in rapidly growing systems that require DNA synthesis and inactive in systems that mostly require energy metabolism. In spite of this altered flux pattern, robustness of the system to variation in environmental and genetic variation is not altered.

Analysis of system population models can reveal underlying robustness mechanisms

In FMOC there is an interesting mechanism for robustness that stabilizes the level of homocysteine (Hcy). Hcy is significant because numerous epidemiological studies have indicated that an increased level of Hcy (hyperhomocysteinemia) is an important risk factor for cardiovascular disease (Lentz 2001). Homeostatic mechanisms in FMOC are designed to keep Hcy at a low level while allowing adaptive variation in the other reactions in the system. Hcy is produced from methionine (Met) in the methionine cycle by the removal of a methyl group (see Fig. 2). Hcy can be remethylated to form Met by MS, and can be converted to cystathionine by the enzyme cystathionine-β-synthase (CBS), the first step

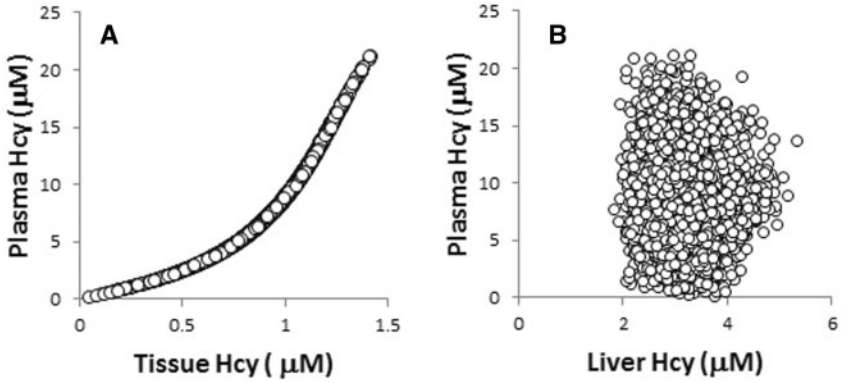

Fig. 4 Dependence of plasma levels of homocysteine (Hcy) on the levels of homocysteine in liver and tissue. These are data from a population of virtual individuals with random variation in all enzymes in FOCM. (**A**) There is a tight but non-linear correlation between plasma and tissue Hcy. (**B**) There is little or no correlation between plasma and liver Hcy.

in the transsulfuration pathway. Hcy is also exported by cells into the extracellular space and the blood (Fowler 2001).

In humans, Hcy is measured in the blood, of course, and so it is a natural and important question to ask what controls the Hcy concentration in the blood. Since one-carbon metabolism occurs in all cells this is not such an easy question. Traditionally it has been assumed that the Hcy blood concentration is controlled by the liver since all amino acids taken up from the gut go there first and the enzymes that metabolize Hcy are more active in the liver than anywhere else. In order to investigate this larger systems question, we took our model of the folate and methionine cycles in the liver (Fig. 2) and modified it so it was appropriate for other tissues where enzyme expression levels are different. We then created a whole body ODE model with a liver compartment, a tissue compartment, and a plasma compartment through which the liver and tissues communicate (Duncan et al. 2013). We took this deterministic whole body ODE model and made a system population model by the methods described above. The virtual population showed distributions of tissue folate, plasma folate, and plasma Hcy nearly identical to those seen in the National Health and Nutrition Examination Survey (NHANES; https://www.cdc.gov/nchs/nhanes/) studies, confirming that the model is a reasonable representation of the underlying physiology (Duncan et al. 2013).

Figure 4 shows several scatterplots of HCY concentrations from the system population model. Figure 4B shows that there is no correlation between liver Hcy (L-Hcy) and P-Hcy. In contrast, Fig. 4A shows that P-Hcy is highly, but nonlinearly, correlated with tissue Hcy (T-Hcy). Analysis of L-Hcy and T-Hcy levels (not shown) shows that they are uncorrelated. The advantage of a system population model based on an underlying deterministic model is that once one sees a phenomenon in the virtual population one can go back to the deterministic model and do computational experiments to determine the causal reasons for the phenomenon. In this case, the BHMT reaction that remethylates Hcy in the liver is not present in tissue and CBS, which transsulfurates Hcy, is only weakly expressed in most non-liver tissues. Thus Hcy builds up in tissues and is exported to the blood. From the blood, Hcy is taken up by the liver. Since the combined volume of body tissues is larger than the volume of the liver and the blood, there is a large net flux of Hcy to the liver. Hepatocytes express BHMT and CBS strongly, so, in the liver the BHMT reaction uses Hcy to make Met, and CBS turns Hcy into cystathionine and sends it down the pathway that makes glutathione. The basic outline of this story has been verified experimentally (Borne et al. 2009). Although this is a simple example, it shows the usefulness of system population models based on deterministic ODE models. By following through on odd or surprising features of the virtual population one can discover new phenomena. In this case, stabilization or robustness of Hcy requires the interactions of the FMOC pathway between tissues and liver and is in effect a higher systems-level property.

System populations models and predisposition to disease

Perhaps the most important thing that robustness mechanisms do is to provide protection from environmental factors and mutations that cause disease. We will use our work on dopamine (DA) metabolism (Best et al. 2009, 2010b; Reed et al. 2009) to illustrate how system population models can give meaning and mechanistic understanding to the

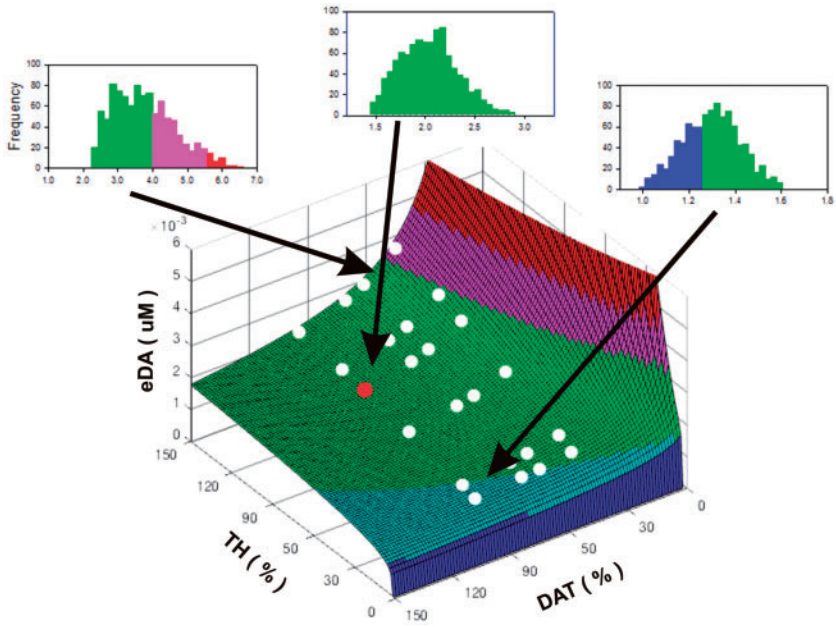

Fig. 5 Dependence of extracellular dopamine (eDA) on the activities of tyrosine hydroxylase (TH) and the dopamine reuptake transporter (DAT). The phenotypic landscape graph has a large homeostatic plateau, indicated by green, where there is little variation in the level of eDA. The red dot indicates the position of the normal wild-type activities for TH and DAT (scaled to 100%). The white dots are known polymorphisms for these two peptides (data in Nijhout et al. [2015]). Some of these are near the downward cliff of low eDA and exhibit dystonias and symptoms of TH deficiency. The upward cliff is associated with anxiety disorders and hyperactivity. Cocaine acts by blocking DATs and would thus move an individual up that slope. Bar graphs are frequency distributions of extracellular DA levels of virtual populations centered at the locations indicated by the arrows. Color coding is as in the surface graph.

concept of "predisposition to disease," which emerges naturally from robustness mechanisms.

Dopamine and serotonin are unusual neurotransmitters in that the system attempts to maintain a constant concentration in the extracellular space, a phenomenon known as volume transmission (Best et al. 2009). If levels rise above or fall below a target level, there is a change in the activity patterns of downstream neurons associated with a change in behavior. For instance, reduced levels of DA lead to torpor, the loss of motor control, and tremors characteristic of Parkinson's disease, whereas elevated levels lead to impulsive behavior and activate the pleasure and reward system. Reduction in 5HT levels is associated with depression and other affective disorders. DA and 5HT levels are maintained by a balance between synthesis and breakdown by monoamine oxidases (MAOs), and by a homeostatic mechanism that involves two kinds of feedback. First, DA and 5HT are continually released from synaptic vesicles and then transported back into the presynaptic neuron by reuptake transporters. Second, these neurotransmitters act on presynaptic receptors (autoreceptors) whose activity inhibits both their synthesis and release. Thus when DA (or 5HT) levels in the extracellular space rise, reuptake activity is increased and synthesis and release are inhibited, and when the levels fall the reverse responses take place.

Models of DA metabolism and regulation have shown that these mechanisms stabilize extracellular DA against mutational variation of the activities of genes in this system (Best et al. 2009; Reed et al. 2009). This can be seen by drawing a graph of the dependence of extracellular DA levels on the activities of tyrosine hydroxylase (TH) and the reuptake transporter (DAT), shown in Fig. 5, graphed with normal activity scaled to 100%. The surface is very nonlinear with a large flat region where variation in TH and DAT has little effect on the DA levels. The large white dot on the surface is wild type and the color coding is green for values considered "normal" and other colors for excessively low or high values of extracellular DA that are associated with symptoms of neurological disorder. It turns out that in human populations there are common genetic polymorphisms of TH and DAT, whose effect is shown by the smaller dots in Fig. 5. These mutations have large effects on the activities of these gene products, yet they all lie on a relatively flat part of the plateau and thus have little effect on the phenotype. This is another example of cryptic genetic variation. Statistical association studies in this system as well as FOCM have

shown some of these polymorphisms to be associated with a "predisposition" to disease (Knappskog et al. 1995; Thomas et al. 2009).

Other mutations farther upstream can also cause a reduction in TH activity and produce the so-called TH deficiency syndrome (TDS). TDS is a spectrum disorder, meaning that symptoms can range from very mild to very severe (Furukawa and Kish 2014), presumably because there are additional contributing factors that vary individually. We can study these phenomena with a population model version of dopamine metabolism in which we introduce individual variation in other components of the system. In Fig. 5 we show, as bar graphs, population distributions of extracellular DA centered on three genotypes with normal, low, and high TH activity.

All individuals in a variable population, centered on the wild type genotype, have normal extracellular DA, indicated by the green color. This is the advantage of having a wild type genotype that sits in the middle of the homeostatic plateau. Despite substantial variation in all parameters, everyone in the population has normal DA. It can be seen that even with populations centered near the upward or downward cliffs, a large number of individuals still fall with within the normal range of extracellular DA, thanks to the homeostatic mechanism. However, a substantial fraction of the population has excessively low or high extracellular DA levels. Low DA levels are associated with neurological disorders such as Parkinson's disease, and there is a hypothesis that hyperactivity of dopamine transmission underlies many of the symptoms of schizophrenia (Kegeles et al. 2010). The fact that amphetamines, cocaine, and other drugs that increase levels of extracellular DA by blocking DAT cause similar symptoms to schizophrenia supports this hypothesis.

We see that even with a genotype that puts one at the edge of a disease cliff many individuals will still be "normal." We interpret this variation as indicative of having a "predisposition" to disease, because whether or not a disease will develop, and the degree of its expression, depend on all the other factors in the system. Predisposition to disease can thus be a manifestation of living on the edge of robustness.

Subpopulations with special properties can be identified

If predisposition to disease means living at the edge of a homeostatic plateau, then it would be useful to know if it is possible to identify the subpopulation of individuals that are more at risk than others. In addition, individuals in a genetically diverse population often react differently to environmental factors or drugs. Using system population models, we can identify the characteristics of such subpopulations and using the underlying mathematical model we can determine the reasons why they react differently. We will give an example from serotonin (5HT) metabolism in the brain using an updated version of the mathematical model introduced in Best et al. (2010b). The general structure of the model is similar to the model of DA, except that 5HT is made from tryptophan (instead of tyrosine), it is hydroxylated by tryptophan hydroxylase (TPH), instead of TH, and it is taken up from the extracellular space by serotonin reuptake transports (SERTs), instead of DATs. As in the case of DA, the concentration of 5HT in the extracellular space is stabilized against polymorphisms in TPH and SERTs by the 5HT autoreceptors (Best et al. 2010a, 2010b).

With this model we can study the differential effects of different antidepressants. MAO inhibitors are antidepressants that inhibit the enzyme that catabolizes 5HT in the cytosol. The idea is that this should raise the cytosolic concentration of 5HT and therefore also the vesicular concentration, and more 5HT should be released per action potential. This should raise the concentration of 5HT in the synapse. Selective serotonin reuptake inhibitors (SSRIs), by contrast, are antidepressants that inhibit the SERTs, thus slowing reuptake from the extracellular space into the cytosol, and this should also raise 5HT in the extracellular space. There are lots of natural questions. Which type of antidepressant works better? On which members of the population? What happens if both are prescribed? Here, without giving details, we'll just show how one can get at these questions using system population models.

We made a system population model of 300 individuals in which MAO activity and SERT varied independently with a uniform distribution with standard deviation 50% of the normal activity (scaled to 1). Figure 6A shows the scatterplot of extracellular 5HT (EHT) versus the activity of SERT. The green dot is the normal steady state. The red dots are those members of the population that have approximately normal SERT values (between 0.8 and 1.2) and quite low MAO values (less than 0.4). As one can see, inhibiting MAO does raise 5HT but not dramatically. In Panel B, the red dots are the members of the population that have approximately normal MAO (between 0.8 and 1.2) but quite low SERT activity (less than 0.4). As one can see, these individuals have significantly raised 5HT.

What would happen if both MAO and the SERTs are inhibited, as might occur under treatment with

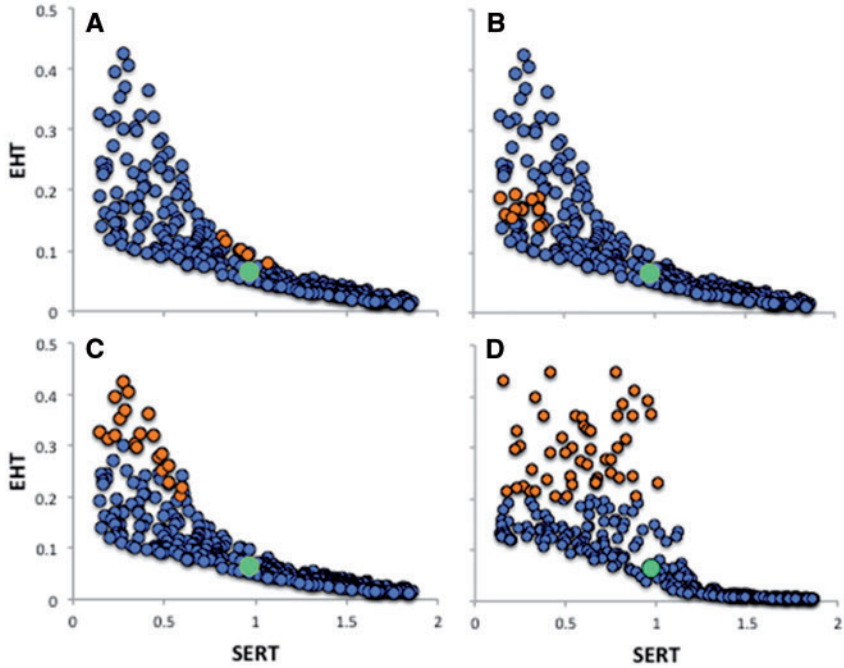

Fig. 6 Dependence of extracellular 5HT (EHT) concentration on the activity of the serotonin reuptake transporter (SERT) and monoamine oxidase (MAO) activity. In all panels the green marker indicates the normal wild type mean, and the red dots indicate 5HT concentrations under the following conditions: (**A**) normal SERT and reduced MAO; (**B**) reduced SERT and normal MAO; (**C**) reduced SERT and reduced MAO. In (**D**) we recreated the population varying SERT and MAO after removing the effect of the autoreceptors. There is a much larger variance and many more individuals show very high extracellular 5HT.

both types of antidepressants? In Fig. 6C the red dots are the members of the population who have relatively low MAO (less than 0.6) and relatively low SERT (less than 0.6). Now we see a dramatic average rise in 5HT with many members of this cohort showing 5HT increases by more than a factor of 4. It is known that the MAO inhibitors can act synergistically with SSRIs and produce dramatic (sometimes life-threatening) increases in 5HT, known as serotonin syndrome (Izumi et al. 2006; Lane and Baldwin 1997), and we see that effect here.

Finally, we examined the importance of the autoreceptors in stabilizing 5HT levels. We created a new population with MAO and SERT varying, but with the autoreceptors turned off. Figure 6D shows the result. There is much more variance in the 5HT levels in the populations and some individuals would show serotonin syndrome even at relatively normal values of SERT. And, one can see how low 5HT gets for high SERT activities. This demonstrates the importance of the autoreceptors in stabilizing 5HT levels.

The mechanisms by which antidepressants such as SSRIs exert their therapeutic effect are not clear. A given drug is only effective in a fraction of the patients and its efficacy is variable (Jia et al. 2016). We can use a system population model of 5HT signaling to study how a population of virtual individuals with a broad range of variation in all parameters responds to an SSRI treatment. This may allow one to determine what underlying factors control the variability in the response.

Conclusions on systems plasticity and robustness

Although homeostatic mechanisms can be very good, they are never perfect. The region of parameter space over which they work is limited, and the homeostatic plateau is usually slightly tilted so that there is actually slight phenotypic variation due to genetic and environmental factors. Thus robustness is not absolute. But homeostatic mechanisms, by their very nature, must also contain explicit mechanisms for plasticity. This is because in order to keep a target phenotype near its setpoint it is necessary to have variables that counteract deviations from that setpoint, in both directions. In a dynamically stable phenotype there are always factors that vary in order to maintain stability. So, the overall system may show stability or robustness in the face of perturbation, even while the underlying components are showing plasticity. The seeming paradox of both

stability and plasticity in the regulatory system therefore stems in part from differences in the "level of analysis" selected by the researcher.

Using mathematical models of metabolic systems it is possible to explicitly understand actual mechanisms of robustness that operate in specific networks, and identify specific genes whose effect is buffered by that mechanism and thus constitute cryptic genes.

We illustrate this with specific examples from FMOC. One of the functions of the feedback mechanisms in FMOC (Fig. 2) is to stabilize several reactions against genetic and environmental variation (Nijhout et al. 2004, 2006b, 2008). These reactions are thymidylate synthase (TS), the rate limiting step in DNA synthesis, AICART, necessary for the de novo synthesis of purines, and DNA methyltransferase (DNMT), necessary for timely methylation of newly synthesized DNA. We showed using our model for FMOC that when we introduce variation in amino acid input, simulating daily meal patterns, that many of the reactions fluctuate widely, some even reversing direction, after and between meals. At the same time, the three reactions mentioned above remain quite stable. We also showed that inactivating one or more of the feedback reactions in the model can result in the destabilization of one or more of the three critical reactions (Nijhout et al. 2014, 2015; Nijhout and Reed 2014).

The plastic reactions that respond to variation in amino acid input are examples of plasticity in service of robustness. These variable reactions also cause their neighboring metabolites in the network to fluctuate widely. Some of those metabolites act as allosteric activators or inhibitors of enzymes elsewhere in the system (Fig. 2), and alter their activity. The allosteric sites on those enzymes are due to particular genetic sequences that can evolve, or can be translocated to other protein coding genes whose product can then come under novel control. Presumably this is how the complex of regulatory interactions illustrated in Fig. 2 originated during evolution.

Rather than focus on principles, we have used specific examples of metabolic systems related to human health to illustrate mechanisms of robustness, plasticity, and cryptic genes. Many of the mechanisms (e.g., feedback and parallel pathways) belong to categories that are well-known, others (e.g., substrate inhibition) are not. Robustness is a systems property, not a function of "robustness genes," and thinking about genes as enhancing or diminishing robustness is unhelpful in understanding both the mechanisms and evolution of robustness. A particular advantage of our approach is that we can account for the roles of specific genes in the mechanisms of robustness. This could be a starting point for uncovering the pathway by which these mechanisms evolved.

Funding

Supported by grants IOS-0744952, EF-1038593, IOS-1562701 an IOS-1557341 from the National Science Foundation.

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