Cell

Beyond Molecular Codes: Simple Rules to Wire Complex Brains

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http://dx.doi.org/10.1016/j.cell.2015.09.031

Molecular codes, like postal zip codes, are generally considered a robust way to ensure the specificity of neuronal target selection. However, a code capable of unambiguously generating complex neural circuits is difficult to conceive. Here, we re-examine the notion of molecular codes in the light of developmental algorithms. We explore how molecules and mechanisms that have been considered part of a code may alternatively implement simple pattern formation rules sufficient to ensure wiring specificity in neural circuits. This analysis delineates a pattern-based framework for circuit construction that may contribute to our understanding of brain wiring.

Introduction

The brain, as we neuroscientists like to say, is *really* complex. A good deal of our efforts are therefore dedicated to figuring out just how this apparent complexity is generated: where does the information to build a brain come from, and how is such information turned into synapse-specific wiring? We call this the ''brain wiring problem.''

We know how it is not done. For example, there cannot be a genetic blueprint that describes every synaptic connection in the way a blueprint of a microchip or electrical wiring diagram does (Figure 1A). Why is this? A blueprint can clearly be envisioned that precisely matches any given neural circuit, allowing for it to be reproducibly built. However, the complexity of such a blueprint exactly equals the complexity of the actual wiring diagram. In other words, this solution generates a new problem that is just as difficult: How is the blueprint generated? It is like answering the question of how life evolved on earth by arguing that it may well have arrived here from a different planet. This is indeed a solution, but an unsatisfying one because it leaves the equally difficult and interesting question of how life evolved on some other planet unresolved.

In this Perspective, we will discuss the concept of developmental algorithms as a solution to the problem of how only a few thousand genes can produce complexity in brain wiring. We will begin by defining some of the key terminology. Next, we consider the evidence for deterministic molecular codes that could define connections, akin to blueprints defining electrical circuits. We will then discuss examples of molecules that were once envisioned to be part of a code but were subsequently revealed to execute developmental rules that integrate stochastic processes in the development of neural circuits. These examples highlight how complicated structures can be generated through simple pattern formation rules rather than elaborately defined addresses. In the last section, we will discuss the difference between an understanding of brain wiring in terms of developmental rules versus the more common focus on mechanisms of individual molecules that execute those rules.

Genetically Encoded Stochastic Invariability?

In order to understand to what extent and how genes can encode brain wiring, we need to first establish a few simple definitions. First, we define developmental outcomes as ''genetically encoded'' if environmental input does not contribute any instructive information to that outcome. A common assumption is that a genetically encoded process is invariable, but this would be a misunderstanding. For example, grafting two branches from the same apple tree on two different root stocks will generate two genetically identical trees with non-identical branching patterns—just as no two dendritic arbors of genetically identical Purkinje cells ever look exactly the same (Figure 1B). A simple algorithm can generate an invariable branching pattern (Prusinkiewicz and Lindenmayer, 1990) (Figure 1C); however, introduction of random inaccuracies can create variability in such a system (Figure 1D). Small environmental differences undoubtedly play a role in generating the differences observed in branching patterns between genetically identical specimens. However, this type of differential environmental input does not contain information for any specific branching pattern; rather, the developmental program ensures robustness of functionally important aspects and allows for variability otherwise. By our definition, such a process is genetically encoded, because the environmental input did not contribute any instructive information to generate that outcome: in both the apple tree and the Purkinje cell, the precise position of branches is irrelevant as long as the final branched structure covers a certain region in a specific manner. Hence, genetically encoded developmental algorithms can produce similar but non-identical structures in the brain.

Small environmental differences are a random variable and therefore define a stochastic process that can lead to variability in the outcome. Conversely, a system is defined as deterministic

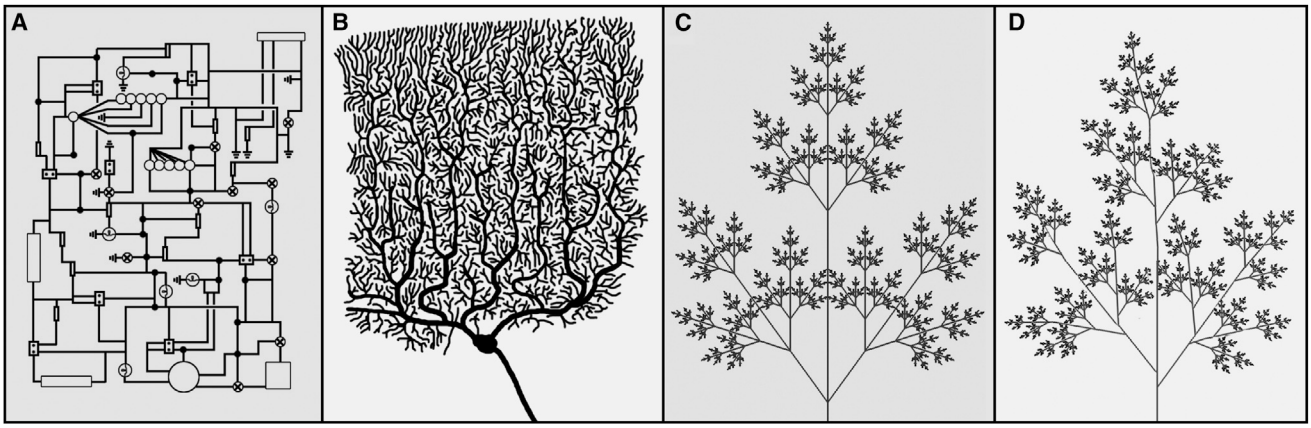

Figure 1. From Deterministic Blueprints to Stochastically Branched Structures in Biology
(A) Schematic of a hypothetical electrical blueprint with deterministic definition of all contacts.
(B) Schematic drawing of a Purkinje cell after a well-known drawing from Ramón y Cajal. The precise branching pattern, number, and placement of dendritic endings is variable.
(C) A simple computer-generated branched structure. The deterministic definition of all branches is generated by a few lines of code (Lindenmayer system) using L-Studio 4.2.13 by Przemyslaw Prusinkiewicz and Radek Karwowski.
(D) The same branched structure as in C, but with stochastic pattern changes.

if no randomness is involved in the development of future states of the system. However, invariability in the outcome does not require a deterministic system. As we shall see throughout this Perspective, even when the outcomes appear invariable, the processes that generate them are often stochastic. All genetically encoded developmental signaling events contain stochastic processes. Notch signaling provides an excellent example. This pathway encodes a molecular mechanism that breaks the symmetry in cell differentiation by ensuring that only one daughter cell becomes cell type A, the other type B. The outcome of exactly one cell type A and B is invariable, but it is crucial to consider that the fate from the perspective of the individual cell is in fact stochastic, because it is impossible to predict which of the two cells will be A and which will be B. We call this a genetically encoded stochastic process with an invariable, or highly stereotyped, outcome. Thus, be it during the development of the vertebrate heart (de la Pompa and Epstein, 2012) or the fly eye (Carthew, 2007), stochastic processes are parts of genetically encoded developmental programs that lead to highly stereotyped and robust outcomes.

A particularly insightful example of a developmental process critical for brain wiring is the idea that ''cells that fire together, wire together'' in the mammalian visual system (Shatz, 1996). This process is based on spontaneous activity waves that Shatz and colleagues first saw sweeping over the ferret retina even before these cells are capable of receiving environmental input. While the activity waves are stochastic, they result in stereotyped layer formation in the lateral geniculate nucleus. Some variability occurs (e.g., the precise size of the layers), but no environmental input contributes instructive information to generate the developmental outcome. Hence, we consider this process a genetically encoded stochastic process with a highly stereotyped outcome. As an aside, it follows from these considerations that an ''activity-dependent'' process can be part of a genetically encoded program. In this Perspective, we only explore such genetically encoded processes, while the important roles of environmental input in activity-dependent synaptic fine tuning are reviewed elsewhere (Ganguly and Poo, 2013; West and Greenberg, 2011). Obviously, genes do not encode stochastic spontaneous neuronal activity just as they do not encode stochastic branching patterns. Instead, in both cases, gene activity defines the developmental algorithms that lead to such cellular behaviors.

An important lesson from these examples is the use of stochastic processes as an integral and necessary part of developmental algorithms, which contrasts with the view of noise as something that development just has to cope with or minimize in order to create a robust outcome (Clarke, 2012; Melé et al., 2015). Recent work in several fields has highlighted the importance of understanding both stochastic processes and heterogeneity of cellular behavior. These fundamental features of all biological systems are lost when we focus on studying averages (Altschuler and Wu, 2010; Losick and Desplan, 2008). How developmental algorithms generate apparent complexity in brain wiring therefore requires insights into the developmental process that are often non-intuitive and quantitative.

Molecular Codes and the "Complexity Reduction Model"

What fundamental solutions to the brain wiring problem do molecular codes offer? Genes encode molecules, and molecules can theoretically provide combinatorial codes of almost any complexity. The success story of molecular biology and gene discovery provides us with ample examples. The obvious candidates for establishing molecular codes in intercellular, synapse-specific interactions are secreted and membrane-associated ''guidance cues,'' their receptors, and cell adhesion molecules (Kolodkin and Tessier-Lavigne, 2011). These cues include molecules that belong to ''canonical'' guidance cue families (Netrins, Slits, Semphorins, and Ephrins) as well as cell adhesion molecules of the immunoglobulin or cadherin superfamilies. The

canonical secreted and membrane-associated guidance cues function at long range or short range to mediate attractive or repulsive signals; cell adhesion molecules may function through direct contact-mediated homophilic or heterophilc interactions. These molecules and mechanisms are reviewed in detail elsewhere (Kolodkin and Tessier-Lavigne, 2011; Raper and Mason, 2010; Yogev and Shen, 2014).

Many of these genes can produce mRNAs resulting from different splice variants or utilization of multiple promoters, further increasing the numerical potential for different combinatorial codes. The most impressive examples are invertebrate Dscams and vertebrate Protocadherins, since members of each family are present in thousands of different splice variants that are required for wiring specificity (Lefebvre et al., 2012; Schmucker et al., 2000; Zipursky and Sanes, 2010). In addition, many of the key growth factor and embryonic patterning pathways such as Wnt, FGF, EGF, and BMP have been found to be required for brain wiring (Charron and Tessier-Lavigne, 2005; Srahna et al., 2006). Thus, a numerically large array of molecular combinations is, in principle, available for neurons to both display and respond to.

A common feature of guidance cues and receptors is their cell-specific expression and spatiotemporally dynamic localization (Chan et al., 2011; Williamson et al., 2010; Zschätzsch et al., 2014). This observation further supports their potential roles in establishing molecular codes; temporal coding using the same guidance receptor can generate a "code in time" to specify target areas (Petrovic and Hummel, 2008; Yogev and Shen, 2014). Hence, a picture emerges in which different cells may express distinct combinations of guidance cues and receptors; different combinations of these molecules can then be presented at distinct places and at specific times during brain wiring to provide unique targeting and synapse formation signals. It is also often argued that early connectivity events take place in much less complicated wiring environments than the final pattern might indicate. The resulting model thus assumes a stepwise process successively restricting possible targeting choices such that the problem of choosing among thousands of options may never occur. We call this the "complexity reduction model."

Numerous elegant mechanisms that contribute to complexity reduction have been put forth. For example, classic guidance cues can form gradients that help in the parallel targeting of many axons simultaneously (Kolodkin and Tessier-Lavigne, 2011; Yogev and Shen, 2014). Parallelized targeting creates repetition and thus redundancy in information encoding: a molecularly encoded blueprint for such a system only needs to specify one of the repetitive units. In addition, even a limited number of guidance cues may be consistent with the apparent complexity of brain wiring if specificity is achieved through regulation of guidance cues in space and time, through protein expression levels, or through the utilization of heteromultimeric receptor machinery. The complexity reduction model is thereby proposed to provide a solution to the brain wiring problem: we may not yet have worked out all the details of when, where, and what kinds of combinatorial molecular codes occur, but there seems to be no fundamental problem. Or is there?

The Molecular Code and Its Discontents

After establishing the idea of the complexity reduction model, we must ask how the underlying stepwise, spatiotemporal code is generated. How do specific combinations of guidance cues selectively and precisely get to be at the right time and place to function as meaningful synapse specification signals? Here again, we face the blueprint problem: the establishment of a deterministic, spatiotemporally precise molecular code would be a problem of comparable complexity to the wiring diagram that it is supposed to explain.

The blueprint problem holds irrespective of whether the code is determined through numbers of molecular cues, temporal control of molecular cues, or differential expression levels of molecular cues; it is not obviously easier to control the precise proteins levels of one cue in space and time than to control the precise combination of several cues only in space (Chan et al., 2011). The example of gradients highlights one way to create repetition of similar structures and thereby reduce the amount of information, or codes, needed. Complexity can thus theoretically be reduced through the exact repetition of a precise and deterministic address code system. However, repetitive structures in brain wiring do typically, and maybe without exception, allow for some level of variability, revealing an underlying stochastic process. This variability may be functionally irrelevant (like differences in precise branching patterns, comp. Figures 1B–1D), but it can reveal the underlying developmental rules and mechanisms: molecular cues that provide approximate guidance for many axons in parallel do not specify a precise one-to-one address code. Hence, variability may not just be due to the repetition of a slightly imprecise address code but, rather, an inherent outcome of a stochastic process that neither requires nor generates an address code.

The complexity reduction model allows for variability by making guidance cues less precise. But how does lack of precision ensure robustness of the developmental process? We have already argued that noise is not simply an artifact that biology has to "live with" and try to reduce but is an integral part of how a developmental algorithm functions in brain wiring. How do guidance molecules deal with or even utilize noise? Numerous influential studies on cell adhesion and guidance receptor functions in different model systems provide us with ample examples for a new and surprising understanding of what these molecules do. For example, we now know that, in *Drosophila*, an individual neuron's choice of one out of thousands of Dscam1 isoforms is indeed unpredictable (Miura et al., 2013). Interestingly, a wealth of groundbreaking work on Dscam1 has revealed a function for this non-deterministic isoform choice that is quite different from a precise address code: both Dscam and Protocadherins in vertebrates serve a primary role in mediating self-avoidance, which requires distinction between self and non-self cell surfaces (Lefebvre et al., 2012; Zipursky and Sanes, 2010). Similarly, cell-intrinsic stochastic recycling of EGFR in the growth cones of higher-order visual system neurons in *Drosophila* is required to form a highly stereotyped axonal branching pattern (Zschätzsch et al., 2014). Variability does not arise in these examples by making a molecular code less precise. Instead, variability is a necessary outcome of pattern formation processes following simple rules. Specifically,

Dscams and Protocadherins implement a simple pattern formation rule without providing a "cue" for axon targeting or synapse specification (Kise and Schmucker, 2013; Zipursky and Sanes, 2010). Large numbers of randomly chosen isoforms serve this function, similar to randomly coded remote garage door openers; as long as there are enough different isoforms, the likelihood is sufficiently low that your neighbor's system has the same random recognition code as you do. This beautiful role of the many isoforms of Dscams and Protocadherins is more akin to a pattern formation process than a molecular synapse specification code.

The idea of "non-cue" functions of guidance molecules is further highlighted by the identification of cell-intrinsic functions. If no target is involved in an intrinsic axonal targeting or branching choice, then the implicated molecules cannot function as external cues or address code (Petrovic and Schmucker, 2015). Examples include neuropilin-1 and semaphorin-3a in the mammalian olfactory system (Imai et al., 2009), the protocadherin Flamingo in the fly visual system (Schwabe et al., 2013), and, indeed, Dscam1 in mechanosensory neurons (He et al., 2014). Variability in the outcome arises not from an imprecision of a molecular code but because these processes are intrinsically noisy and because they generate patterns, rather than specify connections.

A picture emerges of how developmental algorithms can "encode" synaptic specificity in neural circuits. The question thereby is: what exactly are these developmental rules, and how can they explain wiring specificity in the nervous system?

The Simple Rules that Can

Any comprehensive mechanistic explanation for brain wiring must explain three fundamental characteristics in a single conceptual framework: wiring specificity, wiring variability, and the inclusion of stochastic processes during development. A rules-based framework does just that. Variability in neuronal branching patterns and neural circuit architecture offers a first glimpse into underlying developmental rules that generate complicated connections. For example, a set of simple rules that includes spacing between axons and self-avoidance can explain both the robust and variable properties of axonal targeting patterns. A classic and fundamental simple pattern formation rule is lateral inhibition, which is often molecularly implemented through Notch signaling. Indeed, recent work in *Drosophila* shows that this classical pattern formation principle in cell differentiation is actively employed by axons of postmitotic neurons during neural circuit assembly the *Drosophila* brain (Langen et al., 2013). Here, stochastic patterning leads to spacing with a defined average between neighboring axons without specifying either the precise number or distance between them. This is an example of a simple rule that does not by itself specify a synaptic connection but must function as part of a larger developmental algorithm.

In another recent example, the seemingly complex wiring principle of the *Drosophila* visual system known as "neural superposition" was computationally modeled based on three simple rules (Langen et al., 2015). Rather than explaining the simultaneous targeting of ~5,000 growth cones through mechanisms of attractive and repulsive molecular cues, the entire synaptic specification process can be explained through pattern formation principles executed as a concatenation of simple genetically encoded subprograms (Hiesinger et al., 2006; Langen et al., 2015). This example presents all features discussed here: wiring specificity (almost error-free connectivity), variability in the precise spatial placement of pre- and postsynaptic elements (which may or may not be functionally relevant), and stochastic processes throughout brain development from neuronal cell fate choice to growth cone dynamics. Importantly, previously identified roles of cell adhesion molecules in this process fit seamlessly into this framework as implementers of pattern formation rules, rather than molecular address codes.

An important aspect of brain wiring models based on simple pattern formation rules is the "sorting together" of presumptive synaptic partners, such that synapse formation is likely to occur between meaningful partners (Hiesinger et al., 2006). Indeed, it is a curious fact that the actual process of synapse formation appears to be astonishingly non-specific across species. Neurons that innervate incorrect target regions generally will form synapses wherever they end up, however wrong the targets. In fact, if given no other choice, neurons readily form synapses with themselves (so-called autapses) that are functionally indistinguishable from synapses in the brain (Bekkers and Stevens, 1991). Hence, as an ingredient for synapse-specific brain wiring, non-specific synapse formation only makes sense in the context of a larger developmental algorithm. Specifically, promiscuous synapse formation can be guided by precise sorting of the right partners; this is observed, for example, in the mammalian olfactory system or the fly visual system (Hiesinger et al., 2006; Imai et al., 2009). Alternatively, activity-dependent pruning can function in sculpting specific layers harboring specific synaptic connections, as is observed in the lateral geniculate nucleus of the vertebrate visual system (Shatz, 1996). In addition, developmental rules inconsistent with address codes are revealed by human patient data. Axons from the left eye normally establish connection in the visual areas of the right brain hemisphere and vice versa. In 2009, physicians described the case of a child born without a right brain hemisphere who nonetheless developed both left and right visual fields, in both the lateral geniculate nucleus and visual cortex, in the left hemisphere alone, resulting in near normal vision (Muckli et al., 2009). Taken together, a picture emerges of how a purely genetically encoded developmental program can lead to synaptic specificity in a neural circuit through a concatenation of simple genetically encoded subprograms that employ stochastic processes and allow for flexibility, without the need for an address code.

What simple rules can, together with unspecific synapse formation, create synapse specific wiring? Rules can pattern axonal and dendritic architectures that result in specific synaptic partners. For example, the development of the complicated dendritic tree of a Purkinje cell (Figure 1B) has been modeled and computer simulated using simple dynamic processes, including stochastic terminal branching and retraction triggered by dendritic contact (Fujishima et al., 2012). Based on similar "on-contact" rules, we would like to propose a simple theoretical experiment. This experiment reveals how two simple pattern formation rules can determine synaptic specificity when assuming that synaptogenesis can occur between any presynaptic-postsynaptic contact. We envision a schematic brain structure (Figure 2) organized into layers and columns with dynamically extending

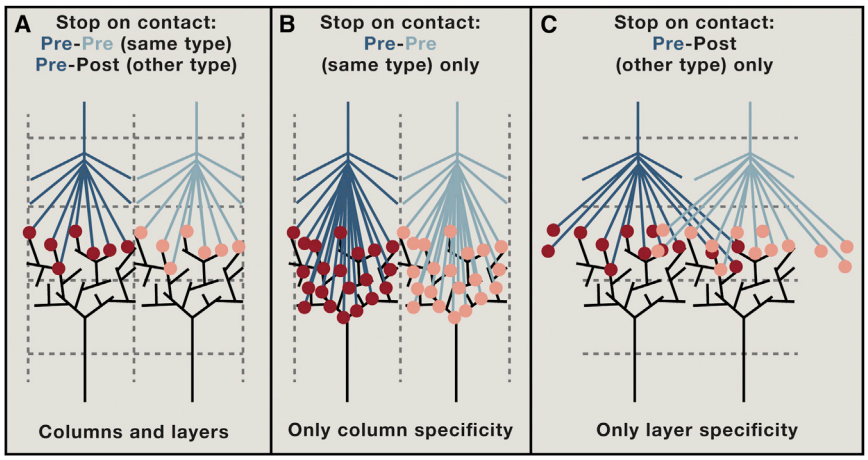

Figure 2. A Theoretical Experiment: Given Promiscuous Synaptogenesis at Any Contact Site, Two Simple Rules Are Sufficient to Generate Layer- and/or Column-Specific Synaptic Contacts

(A) The rule "stop on pre-pre (or same cell type) contact" prevents overlap of neighboring, parallel presynaptic terminals, leading to tiling in columns. The rule "stop on pre-post (or other cell type) contact" prevents overlap within the column; the area where pre- and postsynaptic terminals meet defines a layer. Synapses can subsequently form "unspecifically" between any pre-post contact and are yet restricted to a specific column and layer.

(B) The "pre-pre" rule is sufficient to maintain columns, but without a "pre-post" rule, overlap between different cell types lead to loss of a restricted layer.

(C) The "pre-post" rule is sufficient to maintain layers, but without a "pre-pre" rule, overlap between the same presynaptic cell types leads to loss of columnar restriction.

presynaptic axon terminals (blue) and postsynaptic dendritic trees (black). Synapses (red/pink dots) will form promiscuously at pre-postsynaptic contact sites. In this minimal setup, the two simple rules "stop on presynaptic-presynaptic contact" and "stop on presynaptic-postsynaptic contact" can generate both layer and column specificity (Figure 2A). Specifically, stop on contact between presynaptic axon terminals and dendritic branches restricts the area of synapse formation to a specific layer; stop on contact between presynaptic axon terminals ensures that neither invade each other's column. Correspondingly, loss of the "stop on pre-post contact" rule reduces layer specificity while preserving columns (Figure 2B); loss of the "stop on pre-pre contact" rule reduces column specificity while preserving layers (Figure 2C). A similar observation has recently been made for starburst amacrine cells, which form autapses when self-avoidance is perturbed and fail to form connections with other cells when their contact recognition is perturbed. (Kostadinov and Sanes, 2015). Hence, simple rules such as those executed by cell adhesion molecules previously interpreted as 'guidance cues' or 'recognition codes' can easily generate variety in columnar and layer organization and synaptic specificity. Local interaction rules can be iteratively applied across layers and columns and thereby provide complexity reduction. Finally, the same rule may be executed by different molecules in different systems, as seen for Dscams and protocadherins.

The examples discussed here showcase how simple rules can explain the establishment of synaptic specificity in seemingly complex wiring diagrams to a significant extent. All of these examples are based on iteration of simple rules and thereby create repetitive structures. We speculate that less-repetitive organization can result in brain regions where different developmental algorithms overlap. However, many examples remain where more deterministic solutions based on true guidance and/or matching cues appear to provide a satisfactory explanation. For example, specific laminae in the vertebrate retina are thought to be defined by distinct guidance receptors (Matsuoka et al., 2011). In the *Drosophila* olfactory system, the Teneurins *ten-m* and *ten-a* are proposed to function as homophilic "match-making" molecules (Hong et al., 2012). Importantly, the idea of match-making poses some significant constraints on stochasticity, as both pre- and postsynaptic sides must have "matching" molecular partners and thus deterministic molecular recognition pairs. As a general mechanism for synaptic specification throughout circuit assembly, the idea of match-making is not easily reconciled with stochastic developmental processes. On the other hand, the match-making roles found for these two proteins occur very late in the developmental process to distinguish between few targeting choices prior to synapse formation; thereby, match-making can be understood as an elegant terminal subprogram of a larger developmental algorithm that leads to synaptic specificity.

On the Relation between Developmental Rules and Molecular Mechanisms

Much insight into neural circuit assembly has been gained from single-mutant gene studies that disrupt development. Such experiments are often designed to reveal molecular mechanisms, including attractive or repulsive interactions requiring cell surface receptors during neural circuit assembly. The perspective of developmental rules differs from this approach in the following way: a molecular mechanism executes a developmental rule but may not reveal the rule itself. In contrast, the developmental algorithm is defined as the set of rules that are sufficient to generate robust and precise wiring. Developmental rules can be formulated independent of the molecular mechanisms that execute them, as shown in the theoretical experiment above (Figure 2). More specifically, classical molecular mechanisms of guidance cues and receptors include homophilic and heterophilic binding, both of which can implement either attractive or repulsive responses. But neither of these mechanisms by themselves reveal their roles as guidance cues. For example, the molecular mechanism of Dscam1 (and all its isoforms) is homophilic repulsion; however, this mechanism does not reveal its true role in implementing the simple pattern formation rule of self-avoidance. In contrast, the self-avoidance rule can be quantitatively formulated and understood in the absence of molecular knowledge.

How then can we identify developmental rules independent of the molecular mechanisms that execute them? Curiously, the most common route has remained molecular perturbation

experiments. The assumption in any molecular perturbation experiment is that taking a specific part out will reveal meaningful behavior of the system through the observed response and thus define the function of that specific part. The added hope is that the part whose role is revealed tells us something about the developmental rule that it executed. However, the system is likely to exhibit compensatory responses and secondary effects that may be difficult to interpret. Molecular perturbation experiments are therefore more likely to reveal underlying rules when the perturbation is carried out with high spatial and temporal resolution. For example, analyses of N-cadherin and Flamingo in the fly visual system revealed general principles only through detailed investigation of individual mutant growth cones in relation to identified wild-type or mutant neighbors (Schwabe et al., 2013, 2014).

The characterization of developmental rules independent of the molecules that execute them is probably best achieved through live observation with or without spatiotemporally controlled perturbation. However, the live observation approach demands the ability to observe growth cone behavior and synaptogenesis during a relevant time period in a developing neural circuit without interference and with sufficient spatial and temporal resolution. Where this has been achieved, live observation of individual neurons and their interactions over time yielded important insight into the temporal succession, and thus causal constraints, for underlying brain wiring processes (Langen et al., 2015). In another example, recent live imaging of neuronal migration in the zebrafish retina revealed unexpected cellular behavior leading to amacrine cell lamination (Chow et al., 2015). It will therefore be interesting to extend the live observation to other cell types in the vertebrate retina and the fly olfactory system to see whether cellular behaviors are best explained by code-based target selection mechanisms or simple pattern formation rules.

However we attempt to break down the often quoted ''daunting complexity'' of the brain, a complete solution to the brain wiring problem may bear more similarity to other developmental tissues than our intuition at first suggests. Rules like lateral inhibition, self-avoidance, and gradient-based patterning, as well as underlying mechanisms like heterophilic interaction, homophilic repulsion, and molecular gradients, are well-established facets of the development of all tissues. As such, brain wiring is likely to a large extent an example of particularly complicated developmental patterning rather than a special problem onto itself. While it may seem safest to the engineer in us to explain brain complexity with an equally complicated code, the history of developmental biology teaches us differently again and again: simple rules!

ACKNOWLEDGMENTS

We would like to thank our laboratories and many friends and colleagues for enduring discussions leading to this text. In particular, we want to thank for critical and very valuable discussions and comments on this article: Tom Clandinin, Alex Kolodkin, Dietmar Schmucker, Nevine Shalaby, Steven Altschuler, Lani Wu, and Joris de Wit. Work in the Hassan laboratory is funded by VIB, University of Leuven, FWO (G.0503.12), belspo (WiBrain Network), EMBO, and the European Commission's Marie Skłodowska Curie programs. Work in the Hiesinger laboratory at the Freie Universität Berlin is funded by the NeuroCure Cluster of Excellence, the NIH (RO1EY018884, RO1EY023333), the Muscular Dystrophy Association, the Deutsche Forschungsgemeinschaft (SFB958), and the FU Berlin.

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