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PROBLEMS & PARADIGMS

Prospects & Overviews

Brain wiring with composite instructions

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Abstract

The quest for molecular mechanisms that guide axons or specify synaptic contacts has largely focused on molecules that intuitively relate to the idea of an “instruction.” By contrast, “permissive” factors are traditionally considered background machinery without contribution to the information content of a molecularly executed instruction. In this essay, I recast this dichotomy as a continuum from permissive to instructive actions of single factors that provide relative contributions to a necessarily collaborative effort. Individual molecules or other factors do not constitute absolute instructions by themselves; they provide necessary context for each other, thereby creating a composite that defines the overall instruction. The idea of composite instructions leads to two main conclusions: first, a composite of many seemingly permissive factors can define a specific instruction even in the absence of a single dominant contributor; second, individual factors are not necessarily related intuitively to the overall instruction or phenotypic outcome.

KEYWORDS

brain development, chemoaffinity, genetic background, guidance cue, molecular identification tag, penetrance, permissive mechanism, recognition molecule, Sperry, synaptic specificity

INTRODUCTION

The last three decades have seen great progress in the study of molecular mechanisms that contribute to the development of neural circuits. A key focus has been the quest for molecularly encoded instructions that specify synaptic contacts.^[1–4] In the late 90s and early 2000s, in particular, molecular mechanisms were often categorized as either “instructive” or “permissive.”^[5,6] Candidate molecules are considered more interesting if they function as part of mechanisms that are not only necessary but also sufficient to guide an axon left or right, or to make a synapse or not. Seminal discoveries revealed secreted or membrane-bound ligands and receptors with properties that indicated instructive mechanisms. Soon, the proteins themselves, not just the mechanisms they enabled, were labeled as permissive or instructive guidance cues.^[7–10]

Permissive mechanisms have generally been considered far less interesting. The reasoning is simple: there is a lot of machinery that

needs to be in place to execute an instruction from up top. Disruption of the machinery may prevent an axon from growing, but it does not provide a signal for directed growth or synaptic specificity. Proteins that function in membrane trafficking or cytoskeletal dynamics are obvious examples: they represent basic cell biological machinery required for growth, but they are not part of the instruction for directional growth or selective synapse formation. Or are they?

The terms “instructive” and “permissive” are still in use today, but they seem to have lost some of their edge. Recent reviews use them sparsely or not at all.^[2,11] I know several colleagues who describe them as outdated. If terminology in a field falls out of fashion, it may repay to explore the reasons. Surely, neither the concept of instructive mechanisms, nor the importance of the molecules that execute them have lost any of their relevance. We know that molecules must work together to provide instructions during development. But can a molecule by itself represent an instruction? As typically happens when fields mature, more findings seem to have complicated the matter. Many molecules

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and mechanisms defy the simple, binary categorization. For example, a protein could be considered an “instructive cue” based on the sufficiency of its ectopic expression to change an aspect of synaptic specificity, yet more experiments may reveal that it does so in different ways, or not at all, depending on timing, location, and several other factors.

ARE THERE INSTRUCTIVE MOLECULES?

Molecules and the mechanisms in which they act, underlie all of development. The question is what to pin the label “instruction” to. Is a molecule “instructive,” even if the label only applies to one of its many functions in different contexts? Is a cytoskeletal assembly factor “permissive” even though its activation in a specific context changes synaptic partner choice in a specific manner? Or should the idea be reserved for mechanisms, not individual proteins, because mechanism typically require many proteins to collaborate? Trying to find answers to these questions is not an academic exercise in semantics, but helpful, and may be necessary, to understand how the genome encodes brain wiring through molecular mechanism.

The quest for molecules that represent instructions for directional growth or selective synapse formation has a definite starting point. In the 1930s and 1940s, the mechanisms underlying specificity in brain wiring were thought to predominantly lie in the domain of psychology, learning, and plasticity. Roger Sperry spent much of the 1940s and 1950s revising the prevalent view, including his own PhD and postdoc mentors, to show that brain wiring was a question of genetic encoding and developmental biology.^[12] Sperry postulated instructive molecules. In 1963, he summarized his chemoaffinity theory, which has been a guiding principle of the field to this day: “[...] *the growing fibers are extremely particular when it comes to establishing synaptic connections, each axon linking only with certain neurons to which it becomes selectively attached by specific chemical affinities.*”^[13] Are these “chemical affinities” the molecules that contain the instructions for brain wiring?

Evolution unbiasedly samples the mutational space when programming the brain. Forward screens are the geneticists tool to identify mutations that change brain wiring and its behavioral output.^[14–16] Early screens for behavioral traits have identified mutations in many classes of genes, but not typically cell surface proteins.^[17,18] Twenty years ago, several screens for wiring defects were performed in the fly visual system, including one I contributed to as a postdoc.^[19–22] I remember many discussions across labs about the remarkable variety of genes hit in these screens, which also included, but were not typically enriched for, genes encoding cell surface proteins. What these “mutational space exploration” experiments suggest is that evolution may indeed be able to alter brain wiring in a meaningful, selectable, and heritable fashion based on mutations in a surprising variety of genes. Later work revealed specific contributions of many mechanisms, including membrane trafficking machinery,^[23–25] cytoskeletal regulation,^[26–29] and countless other core developmental mechanisms.^[30–33] How these seemingly permissive mechanisms contribute to the idea of wiring specificity is not immediately obvious. By

contrast, Sperry’s “*chemical affinities*” provided an idea that is both intuitive and provides a testable hypothesis.

Sperry unambiguously proposed what would later be called a molecular key-and-lock mechanism between the axon and its target(s) to the exclusion of wrong connections.^[34] Synaptic connections should not form if the key does not fit the lock. Indeed, over the years, several beautiful examples have been found where molecular interactions strongly favor certain pre-postsynaptic pairings over others.^[2,3,35–37] On the other hand, it has also been found that many, and maybe most, neurons have the capacity to form synapses with incorrect partners when given the opportunity.^[38–43] The notion of promiscuous synapse formation is not at odds with precise outcomes. In fact, in the case of activity-dependent (or other competitive) pruning processes, initially exuberant synapse formation is a developmental requirement for the correct outcome (post-specification).^[38,44–46] Furthermore, only certain cells will get to see each other during development.^[39,47,48] Restrictions in time and space ensure a level of pre-specification that could reach the exclusion of most, and maybe in some cases all, incorrect partners. The more encounters are restricted, the more synaptic promiscuity may be permissible.^[38] In other words, a plethora of molecular and cellular mechanisms are to be expected that function in relevant ways other than molecular matchmaking between presumptive synaptic pairs. And indeed, a plethora of remarkable molecules and mechanisms throughout development have been found that have traditionally been labeled “permissive,” and yet contribute to precision in the outcome. These include processes like dendrite spreading through self-avoidance and other mechanisms that control overlap of axonal and dendritic processes, like tiling and kinetic restriction.^[38,39,49] To what extent the underlying instructions are functions that can be directly assigned to individual molecules, or to what extent the coordinated collaboration of such permissive mechanisms may generate the instructions, are questions that the field has been grappling with to this day. The quest has certainly focused more on the former: the molecules that might go it (almost) alone.

MOLECULES AND MECHANISMS CONTRIBUTE TO COMPOSITE INSTRUCTIONS

Many molecules have been proposed as instructive guidance cues. One of the best-known textbook example for an instructive cue, or “Sperry molecule” is netrin.^[50,51] Netrin was found based on an axon growth assay designed to identify an instructive cue in an otherwise permissive environment.^[50] Mutants for either netrin or its receptor DCC exhibit long-range targeting defects in the spinal cord.^[52,53] However, these early experiments were done in complete null mutant animals. It took more than 20 years until the experimental test of a loss of netrin only in the target region was performed. Surprisingly, loss of netrin only at the place to which it was supposed to attract axons has little effect.^[54,55] Instead, loss of netrin along the growth path revealed pathfinding defects. These findings triggered a debate under what conditions netrin should be classified as a long-range attractant.^[56] This debate lies at the heart of the discussion of the distinction between

instructive versus permissive mechanisms. Arguably, the new studies did not render netrin less important than previously thought, they just revealed that there are more context-dependent facets to netrin function. In other words, the instruction to grow toward the midline requires additional factors and conditions. Specifically, netrin needs to be present along the path the axons are growing—much like a growth factor. However, a growth factor is a classic example for a permissive signal: it needs to be there for growth to happen, just like any other part of the basic growth machinery.

Curiously, the most commonly used chemoattractant to instruct directional growth in culture experiments is NGF—neural growth factor. NGF is not a major instructive target signal during brain wiring. However, axons prefer to grow where NGF is, an observation that led to the discussion of its role in “*chemoaffinity in a broad sense*” already in 1990, before the discovery of netrin’s role in the spinal cord.^[57] Whether Netrin or NGF serve an instructive or permissive function depends on context. The molecules themselves are not instructive under all conditions, but they can be key parts of an instructive mechanism if other factors play their parts and the conditions are right. In the case of netrin’s role in long-range attraction, one key part of that context is localization in time and space. A permissive growth factor can be part of an instructive signal, if either the preceding developmental program (e.g., in the spinal cord) or the experimenter (e.g., in a cell culture assay) ensured that it marks a certain path or target at the right time and place. For netrin in particular, an increasing number of context-dependencies have been described, including other factors like Sonic Hedgehog, different localizations and function of Netrin along the axonal growth path, dependencies on the type of model system or area of the spinal cord, to name a few.^[56,58] In the quest for the mechanisms underlying the instruction named “long-range attraction,” netrin is a key player, a component of a composite instruction (Figure 1). The relative contribution of the individual component is a quantitative measure for how much of the instruction it represents.

In its simplest form, a composite instruction could be defined as the sum of its components where each component provides a relative contribution (Figure 1a). However, individual components are not likely to simply add up. For example, the two factors netrin and its receptor DCC are clearly codependent: if one is absent, the other loses its function as well (marked in red in Figure 1). Similarly, the growth speed of a growth cone can contribute significantly to a turning instruction together with a chemoattractant such that the turning angle is steeper if the growth speed is slower; however, the two relative contributions are not independent, because the contribution of the chemoattractant becomes zero if the growth speed is zero. Hence, dependencies of the components among each other increase the number of possible composite instructions that can be generated by a limited number of contributing components. The composite instruction for a specific axon guidance or synaptic partner choice is unique for a given neuron at a given time. There may be as many different composite instructions as there are neuronal choices we care to analyze. And for each composite instruction we are facing two major obstacles for a quantitative description:

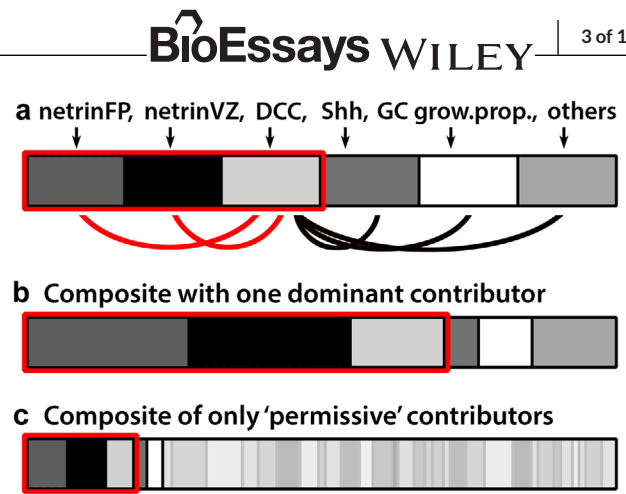


FIGURE 1 Structure of composite instructions. (a) Depicted is a possible composite of the instruction for “long-range attraction,” including known molecular contributions to the instruction (netrin at the floor plate (*netrinFP*), netrin along the path in the ventricular zone (*netrinVZ*), Sonic Hedgehog (*Shh*) and the netrin receptor DCC (*DCC*)). Further contributing components are suggested, including growth cone growth properties (*GC grow.prop.*) and other factors (*others*). The composite instruction is not the simple sum of contributors, because of dependencies of components (indicated by connecting lines). For example, netrin and its receptor DCC depend on each other (marked with a red box). The relative contribution of none of the components is known. (b) shows the example with a dominant contributor; (c) shows the possibility that the composite instruction is entirely composed of small contributors, each of which might be labeled “permissive”.

first, we most likely do not know all components. Second, how to measure the relative contribution of each component?

PHENOTYPIC PENETRANCE AND GENETIC SENSITIZATION ARE MEASURES FOR RELATIVE CONTRIBUTIONS TO COMPOSITE INSTRUCTIONS

It is difficult to know all contributing components for any molecular mechanism. However, not all contributions are equally relevant. The more important a molecule or mechanism is for a composite instruction, the more likely its discovery and characterization in the literature (Figure 1b). At the other end of the scale, there may be countless molecular interactions that each contribute just a little bit to the composite, the genetic background or “context” (Figure 1c). There is no agreed-upon threshold for the measure of relevant versus contextual components.

The relative contributions of known components can be estimated by perturbation analyses. In genetics, phenotype strength and phenotypic penetrance are quantitative measures for the contribution of a genetic aberration to the phenotypic outcome. Loss of components may cause a weaker or stronger phenotype that is identical in all individuals of a population. Alternatively, a phenotype may be more binary (e.g., lethality), but only occur in a certain number of individuals. A combinatorial effect on phenotype strength and penetrance is likely in biological systems. Similar to the relative contributions of individual

components to a composite instruction, the relative contributions to phenotypic strength and penetrance could be summed up for all component perturbations to yield a composite phenotype. However, this simplest possible case is again quickly complicated by dependencies between individual perturbations. Such dependencies are highly likely and increase the number of possible phenotypic outcomes beyond the simple sum.

We observe the consequences of quantitative, partial contributions to composite instructions both in nature and in the lab. In developmental biology, as elsewhere, a mutant phenotype is more interesting if it is “all or nothing” and 100% penetrant. Yet, mutations affecting synaptic specificity are rarely, if ever, of this kind. Some variants of a gene may sensitize a developmental process such that, under optimal conditions, the phenotype may be indistinguishable from wild type. Weakening any other component of the composite instruction will strengthen the phenotypic outcome either by increasing phenotypic strength or phenotypic penetrance, or both. A good example for this is a recent study of Cadherins, a class of cell surface proteins that have been implicated in many phenotypes related to circuit development.^[6,22,59–61] The subfamily of type II Cadherins contains several closely related proteins whose individual genetic loss of function does not cause obvious phenotypes depending on where and how the analysis is done. This gave rise to the hypothesis of functional redundancy and the systematic analysis of double and triple knock-outs for closely related family members.^[59,60,62] For example, a triple knock-out of Cadherins 6, 9, and 10 causes a highly specific defect in the wiring of the retina,^[60] but no defect in the sorting of motor pools in the spinal cord.^[62] The triple-knock-out mice are viable and fertile. With respect to these phenotypes even the triple mutant is sub-threshold for a recognizable phenotypic strength or penetrance. Remarkably, removing the rather unspecifically expressed type I Cadherin N-Cad can cause phenotypes in the spinal cord in the background of a mutant for a type II Cadherin that causes no phenotype even in a double knock-out with its closest type II relative.^[62] These findings exemplify the importance of context for any given mutation.

Figure 2 schematizes the possible contributions of three mutations for a phenotypic outcome for the simplified case of independence and additivity. Based on a threshold for recognizability of strength or penetrance of a phenotype, none of the three mutations may cause obvious defects by themselves (Figure 2a–c) nor in double mutant combinations (Figure 2d–f). Yet, if all three occur together, the strength or penetrance of a highly specific phenotype can reach the threshold (phenotypic peak marked in red in Figure 2g). To the geneticist, these mutations may appear redundant, yet they need not affect the same process at all. Each mutation causes a sensitized background for the other. Each mutation provides context for the other. Subthreshold outcomes of individual mutations increase robustness to perturbation.^[63] Which contribution we regard as “background” and which we regard as “the meaningful perturbation” is an arbitrary, albeit quantitative choice. In the context of mutations A and B, the geneticist may say that mutation C reveals an instruction, because, in that background, the mutation of C takes the instruction away, leading to the phenotype. Maybe only the largest of the three should be labeled as “instructive.” But what if all of

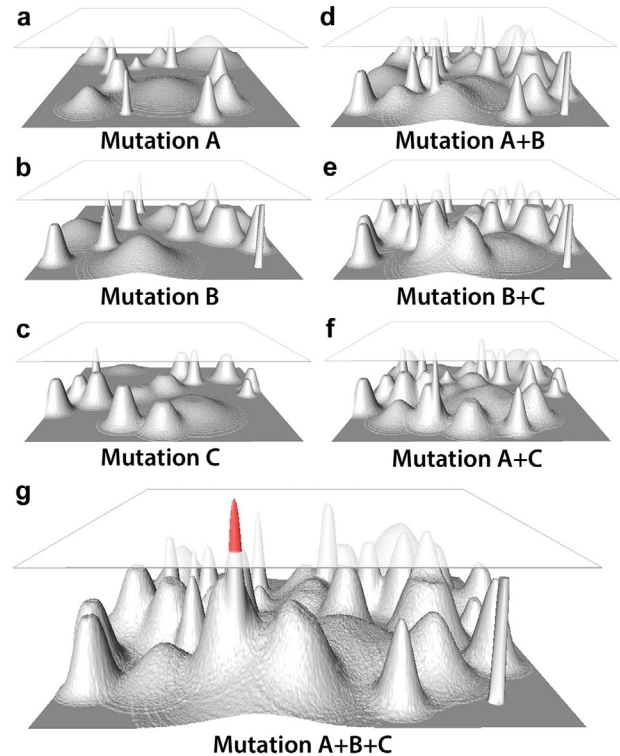


FIGURE 2 How combinations of mutations, genetic background and context can create highly specific phenotypes. Depicted are schematic representations of “phenotypic sensitization landscapes” produced by three mutations and their combinations. The opaque square indicates a threshold for the recognizability of phenotypic strength of penetrance. The depiction is limited to the simplest case of independence and simple additivity of the three mutations. In this example, only the co-occurrence of all three mutations causes a highly specific phenotypic outcome (marked in red). This outcome was not obvious based on the sensitizations cause by any of the single mutations.

a large number of contributing components to a composite instruction are small (Figure 1c)?

Note that the same logic applies to probabilistic phenotypes based on the same genotype as to phenotypic strength. A sensitized background may not only cause no or a weak phenotype, but it may also cause a strong phenotype with low penetrance.^[64] A second mutation may further increase the penetrance, even it does further increase phenotypic strength. Figure 2 can be interpreted in both ways: as a summing of phenotypic strengths, or a summing of phenotypic probabilities. All of this is basic genetics. Basic genetics composes instructive signals based on the contribution of multiple components. If the relative contribution of one component is particularly high, we are more likely to label the component as instructive. On the other hand, if no single component has a particularly high relative contribution, the same instruction may be composed of a large number of components that each can be labeled as permissive (Figure 1c). Yet, in a sufficiently sensitized background, the removal of even the smallest relative contribution can cause a phenotype—an instruction in the context of that sensitized background (Figure 2).

PERMISSIVE FUNCTIONS OF MOLECULAR IDENTIFICATION TAGS CONTRIBUTE TO COMPOSITE INSTRUCTIONS

Some classes of molecules are considered more likely than others to function instructively—or rather as a major contributor to a composite instruction, we should say. Most prominently, molecular interactions of cell surface receptors are preferentially interpreted in the context of identification tags as attractive or repulsive signals, which have traditionally been considered instructive.^[2,3] There is a lot of historic motivation as well as contemporary evidence that puts special emphasis on the role of proteins on the cell membrane in brain wiring.^[2,13,65] Some genetic loci can generate a large variety of cell surface proteins based on alternative splicing, which makes them particularly interesting as potential identification tags. Mutant analyses of such cell surface proteins revealed their important roles in the development of synaptic specificity in neural circuits, yet blurred the distinction between instructive and permissive functions.^[49,66–69] Cell surface protein-mediated interactions between cells can contribute to many remarkable mechanisms during brain development. For example, Neurexin-based trans-synaptic interactions have been shown to play key roles in the instructive specification of synaptic properties, albeit maybe not for synaptic partnerships.^[67,69,70] On the other hand, some of the most elegant and best-studied mechanisms of neuron-specific identification tags found to date turned out to be the least obviously instructive. Both *Drosophila* Dscam and vertebrate protocadherins gained notoriety for their ability to generate many different combinatorial codes, up to thousands, that are cell-specifically employed to distinguish self from non-self.^[49] Twenty years of investigations of Dscam functions opened doors to an understanding of the surprising roles these proteins play during brain wiring, from dendrite spreading through self-avoidance^[71–73] to cell-autonomous functions.^[74] Depending on context, Dscam-Dscam interactions can mediate self-avoidance in one compartment of the cell and function in the dynamic exploratory behavior of axonal sister branches in another compartment,^[74,75] possibly even in the same cell. All of these roles are traditionally classified as permissive. Yet, they are of great importance for the development of synapse-specific brain wiring as components of composite instructions.^[49,72] The realization that one of the biggest successes of the field, and one of the best characterized molecular mechanism of an identification tag to date, turned out to serve exclusively traditionally permissive functions may have contributed to the going out of fashion of the instructive-permissive dichotomy. This notion also resonates in the equally successful quest for molecular target identification tags in the vertebrate visual system since the 90s.^[76] The ephrinA's are ligands for the Eph receptors (EphRs) on the growth cones of the retinal ganglion cell axons.^[77,78] Surprisingly, they were found to function by repulsion rather than attraction.^[79] The repulsive mechanism only becomes an instruction in the context of local gradients, relative positioning of incoming axons in time and an intrinsic drive of the retinal ganglion cells to form synapses where they are not actively prevented from doing so.^[76] Finally, even netrin has in the

meantime been found to exert a function in dendritic self-avoidance in *C. elegans*.^[80] Some permissive functions go a long way, and so do cell surface and secreted molecules as contributors to composite instructions.

More recently, studies on another family of interacting cell surface proteins have revitalized the idea of instructive identification tags. Interacting pairs of 11 DIP proteins and 21 Dpr proteins were found to mark pre- and postsynaptic partners with remarkable specificity in the *Drosophila* brain.^[81,82] Subsequent analyses have identified some of their important roles during development that contribute to synapse-specific wiring. For example, interactions between Dpr6, Dpr10, and DIP- α have been implicated in branch arborization, synapse numbers, and cell survival.^[83] Two other family members, DIP- β and DIP- γ , each have several interacting Dpr proteins (7 and 4, respectively).^[82,84,85] Their loss of function in the fly visual system surprisingly did not reveal a reduction in synapse numbers, suggesting that in their absence, neurons synapse with alternative partners.^[86] Yet, the pair of DIP- γ /Dpr11 has recently been hailed as a “long-sought after Sperry molecule,” again based on a study in the fly visual system.^[87]

There are two subtypes of the UV-sensitive photoreceptor R7 in the fly eye that differentiate probabilistically and form a stochastic projection field in the brain.^[88–90] Each has a specific main postsynaptic partner, two types of the amacrine cell Dm8. How do they find each other? The possible solutions are reminiscent of the discussions in Sperry's early days: either the incoming axons induce the correct postsynaptic cells (similar to Sperry's PhD supervisor's Paul Weiss' resonant theory), or the postsynaptic cells have a predetermined matching identification tag (as Sperry proposed himself). The solution in the fly visual system is both beautiful and remarkable: first, the two Dm8 subtypes differentiate independently of the R7, each with a matching code for one of each presynaptic R7 subtype, as Sperry would have it. However, the cells of both Dm8 subtypes are produced in excess and get into contact with the stochastic field of the two R7 subtypes days before synapse formation starts. Those Dm8s that do not get into contact with an R7 of the matching code are likely to die.^[82,87,91] This observation reintroduces the growth factor theme. Neuronal cell death has also been found in mutants for DIP- α or its two ligands Dpr6 and Dpr10.^[83] In the case of the R7 photoreceptor, the Dpr11 survival signal effectively selects the presumptive synaptic partners during morphogenesis, akin to a highly localized growth factor. The DIP- γ /Dpr11 interaction is arguably a dominant component of the instruction (Figure 3a). Localization of the signal is another key component of the composite instruction. NGF or netrin contributes to composite instructive signals based on their localization along a path or target. Dpr11 contributes to a composite instructive signal based on its localization in a stochastic axon terminal field and its role in providing a survival signal. This remarkable selection mechanism ensures that a field of hundreds of two types of stochastically distributed presynaptic terminals are effectively paired with the correct postsynaptic partner cells long before synapse formation starts.^[87,91] How about partner identification during synapse formation itself?

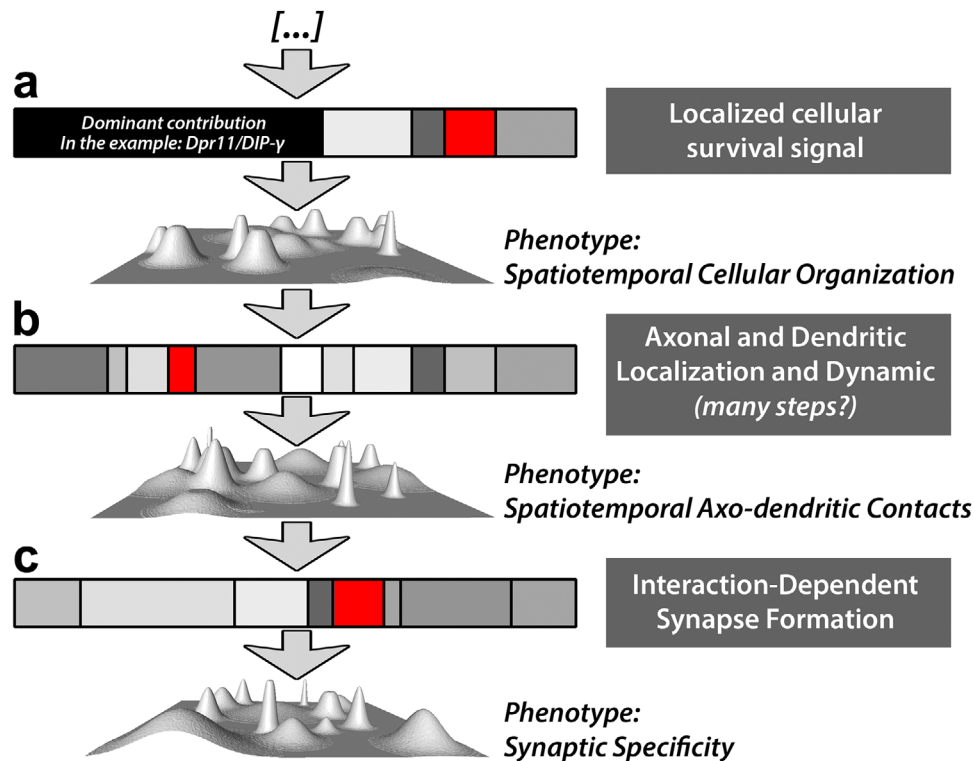


FIGURE 3 Interdependent composite instructions in time lead to synaptic specificity. Depicted is the feedback relationship of composite instructions on intermediate phenotypes that each contribute to the next composite instruction in time. (a) Prior to synapse formation presumptive pre- and postsynaptic partners are brought into vicinity, providing a basis for subsequent developmental steps. A composite instruction for the localized cellular survival of selected cells can be part of this step. In the example discussed in the text, a specific pair of cell surface proteins are a dominant contributor to this composite instruction. (b) Following development of cellular vicinities, composite instructions for axonal and dendritic dynamics further restrict synaptic partner choices. (c) Finally, a composite instruction for the initiation of synapse formation may include many contributors, including biasing cell adhesion and an intrinsic drive to form synapses. Marked in red in all three composite instructions: The same component, for example, a cell surface protein or a ubiquitous enzyme, may contribute as part of several different composite instructions throughout developmental time. Hence, the individual component reveals little about each composite instruction or the phenotypic outcome.

INSTRUCTIONS ARE COMPOSITES IN TIME

The contribution of the DIP- γ /Dpr11 interaction highlights the importance of developmental time as context. The survival signal that leads to the correct spatial arrangement precedes synapse formation by a time period of almost half of brain development during fly pupation. Synapses will form later during development between the pre- and postsynaptic partner cells that have been sorted together during early development. No role of the DIP- γ /Dpr11 interaction during the time period of synapse formation has so far been shown. In fact, loss of *dpr11* does not seem to affect R7 synapse numbers.^[83] It is unclear what synaptic partners R7 chooses in the absence of Dpr11. Interestingly, wild type Dm8 cells extend dendritic projections beyond the column with the correct presynaptic R7 and are capable of forming synapses with the 'incorrect' subtype, albeit rarely in wild type.^[91] This observation suggests that "incorrect" synapses are principally possible, while the spatial vicinity of the correct cells makes synapse formation between the correct partners much more likely. Arguably, the early developmental selection process that ensures spatial vicinity of correct presumptive pairs could permit higher synaptic promiscuity: if only cor-

rect cells are sufficiently close for synapse formation, then the earlier developmental sorting provides a significant relative contribution to the composite instruction for synaptic specificity.^[38] In support of this idea, our group has recently reported a surprisingly prevalent ability of these R7 photoreceptors to form synapses with incorrect partners.^[92]

Drosophila R7 photoreceptor terminals are amongst the first neurons whose live dynamics have been recorded throughout the synapse formation process in intact brains.^[93,94] Loss of autophagic degradation in the R7 cell leads to increased stability of synaptogenic filopodia.^[92] A consequence of this increase in filopodial stability is the formation of synapses with at least six incorrect postsynaptic partner neurons; these are neurons that should not form any synapses with R7 based on electron microscopy-based connectome analysis.^[95] Both autophagy and filopodial dynamics are generally considered permissive mechanisms. Yet, their function in the context of development reveals significant quantitative contributions to the composite instruction for synaptic partner choices. Increasing autophagy does not reduce any specific filopodia in distinct layers or for certain partners only, but instead dials up the dynamics of synaptogenic filopodia across the axon terminus. This results in an effective exclusion of many

postsynaptic partners through fast filopodial kinetics.^[92] In the absence of this kinetic restriction, several incorrect partner neurons are revealed as competent synaptic partners that are not prevented from synapse formation by any other mechanism. Kinetic restriction makes sense as a part of a composite instruction only as a contributing component in addition to other factors and conditions. First, axonal and dendritic processes need to be at the same time and place. DIP- γ /Dpr11-dependent cell death of incorrect pairs is one of the mechanisms that ensure this (Figure 3a). Next, sufficient filopodial numbers and stable encounters must occur between pre- and postsynaptic partners (Figure 3b). And finally, both partners need to be competent to form synapses at the same time. Attractive molecular interactions are likely to bias some partnerships positively, while repulsive interactions may bias against other partnerships (Figure 3c). Each of these steps can be viewed as consecutive composite instructions or a larger composite instruction in time. Importantly, an individual molecule or mechanism may contribute to a different degree to several composite instructions in time (relative contribution marked red in Figure 3). Hence, in different contexts, the same molecule or mechanism can contribute to different instructions. Synaptic specificity in the outcome is a composite based on a sequence of molecular and cellular mechanisms in time.

Are cell surface proteins and secreted ligands more likely to have a higher relative contribution to composite instructions? Based on a literature search for molecules that have been associated with instructive mechanisms it certainly seems so. Cell surface proteins are the molecules that mediate intercellular and trans-synaptic interactions; signals start with the binding of a ligand and are passed down through intracellular signal transduction and cell biological machinery. Efforts have been made to classify cytoskeletal regulators downstream of cell surface proteins as instructive or permissive,^[96] but the origin of the instructive signal remains on the surface. Support for a high relative contribution of cell surface proteins during brain wiring has come more recently from single cell sequencing studies in *Drosophila*.^[2,97,98] For example, developing projection neurons in the fly's olfactory system exhibit significant relative enrichments of transcripts for cell surface proteins and transcription factors.^[97] Notably, the differential expression of cell surface proteins in these neurons peaked in early brain development and decreased during the time of synapse formation, suggesting many roles prior to synaptic partner identification. Another transcriptome analysis of inhibitory cortical interneurons revealed several classes of synaptic proteins as enriched, including cell adhesion, transmitter release machinery, ion channels and growth factors.^[99] A specific set of synaptic proteins, including cell surface molecules, provide a molecular signature for neurons in time and space. These findings, together with a wealth of literature unparalleled for any other class of molecules in brain wiring, are testimony to the many remarkable roles played by cell surface proteins and their ligands as parts of mechanisms that far extend the conceptual boundaries of "matchmaking."

The precise relative contributions of cell surface proteins to the many important mechanisms they are part of is less clear. As discussed above, for a given genetic background a contribution of almost any amount can appear as the decisive factor for a dramatic increase in

phenotypic strength or penetrance. A focus on the role of one class of molecules renders all other contributions "context" by definition. Yet, the composition of an instruction is driven by evolution, unbiased and principally quantitative in nature.^[63] If the molecular function of a gene product seems less obviously linked to brain wiring, it may just be because a relative contributor rarely reveals the function of a composite instruction. No matter what molecule.

CONCLUSIONS AND OUTLOOK

The number of composite instructions that lead to synaptic specificity in time may be as difficult to count as the individual components of each composite instruction (Figure 3). Axon guidance has been a vibrant field in its own right for decades for a good reason: to understand the molecular mechanisms of just a single growth cone decision is a formidable challenge. By the time all the cables are laid, and the final decision has to be made with which partner to synapse, the choices have been reduced dramatically. It is now that the final bias, a molecular match, or the exclusion of the remaining wrong possible partners through molecular repulsion permit the actual formation of a synapse. The developmental sequence of decisions also highlights the ultimate composite of instructions in time.

Looking forward, composite instructions provide a framework to understand the contributions of many molecules and mechanisms that have so far been regarded as less relevant for our understanding of brain wiring. A need for this framework is particularly highlighted by the realization that a highly specific instruction may be composed exclusively of seemingly small, permissive contributors. Intuition about the role of a single factor in isolation may be of limited help to understand a composite instruction. To the contrary, some of the biggest successes in the field started out with the intuition (and testable hypothesis) of synaptic identification tags, yet the underlying molecular and cellular mechanisms turned out to be more varied, and arguably more interesting, than the intuitive hypothesis suggested. And none of these mechanisms can be understood without the contexts that define composite instructions in time.

The final decision to make a synapse in a highly selected environment only makes sense following the developmental history of the instructions that created that final moment. Each of the instructions in time were composites. And each of the components of these instructions may just as well be regarded as permissive, quantitative contributions to the beauty of the final outcome. A brain grown by permission.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CONFLICT OF INTEREST

The author has no conflict of interest to declare.

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