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Review

Synaptic Specificity, Recognition Molecules, and Assembly of Neural Circuits

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Developing neurons connect in specific and stereotyped ways to form the complex circuits that underlie brain function. By comparison to earlier steps in neural development, progress has been slow in identifying the cell surface recognition molecules that mediate these synaptic choices, but new high-throughput imaging, genetic, and molecular methods are accelerating progress. Over the past decade, numerous large and small gene families have been implicated in target recognition, including members of the immunoglobulin, cadherin, and leucine-rich repeat superfamilies. We review these advances and propose ways in which combinatorial use of multifunctional recognition molecules enables the complex neuron-neuron interactions that underlie synaptic specificity.

After neurons migrate to appropriate positions, they form elaborate networks of connections that underlie our mental activities. This process is conventionally divided into three steps—axons find their way to appropriate target areas (axon guidance), they choose appropriate synaptic partners within those areas (synaptic specificity), and finally, they form functional synapses (synaptogenesis).

In this Review, we focus on the least well understood of these three steps, synaptic specificity. To some extent, the difficulty results from the relative complexity of the problem: synaptic specificity often requires that neurites of dozens of neuronal types choose synaptic partners from a similarly large number of potential targets available to them (Figure 1). This contrasts with axon guidance and synaptogenesis in which interactions at any single time are usually between fewer cells.

Fortunately, progress has been accelerating, in large part due to introduction of new high-throughput imaging, genetic, and molecular methods that enable comprehensive descriptions of the cell types that comprise neural circuits, the connections they form, and the recognition molecules they express. As a result, many newly discovered (or at least newly investigated) families of cell surface proteins have been implicated over the last decade as mediators of target selection. Here, we highlight these new tools and new molecules. A companion Review (Honig and Shapiro, 2020) discusses how the structures of recognition molecules determine their specificity. Based on these new discoveries, we propose ways in which combinatorial use of multifunctional recognition molecules underlie synaptic specificity.

Cellular Strategies for Synaptic Partner Choice

A cellular definition of synaptic specificity is required for understanding the molecular mechanisms that we will discuss here. We propose two simple criteria. First, synaptic specificity involves short-range interactions between synaptic partners, when the growth cone of one is within "filipodial reach" of the other and thus able to make contact without net growth. Second, synaptic specificity involves a predictable choice among synaptic partners within a restricted area—for example, presynaptic neuron A exhibits specificity if it forms synapses on postsynaptic cell A′ rather than on B′ (Figure 2A).

How is this specificity achieved? One possibility is that axon guidance mechanisms bring neurites into close proximity with the degree of overlap determining the likelihood of synapses between them, an idea that has been called "Peter's Rule" (Stepanyants and Chklovskii, 2005). There is, however, little experimental support for Peter's Rule and clear violations have been documented (Briggman et al., 2011; Takemura et al., 2015; Krishnaswamy et al., 2015) (Figure 3). These results imply that molecular mechanisms bias connectivity in favor of a subset of equally proximate partners. Among them, the most straightforward is that processes of one cell type bear cell recognition proteins that bind selectively to receptors on the surface of partner cells; recognition leads to an adhesive interaction that favors synapses between appropriate partners (Figure 2B). This is, indeed, a common mechanism and has been the predominant influence on the way we think about the problem.

However, many other intercellular interactions also lead to specific connectivity. First, cell-cell recognition can lead to generation of a repellent signal, resulting in a decreased incidence of synapse formation between neurons that express binding partners (Figure 2C). Second, differential adhesion can act after synapses form, leading to elimination of some synapses and stabilization of others (Figure 2D). Third, interactions of neurons with cells other than synaptic partners can promote specificity. Fourth, in many regions of the nervous system, axons or dendrites arborize in just one or a few of several narrow, parallel

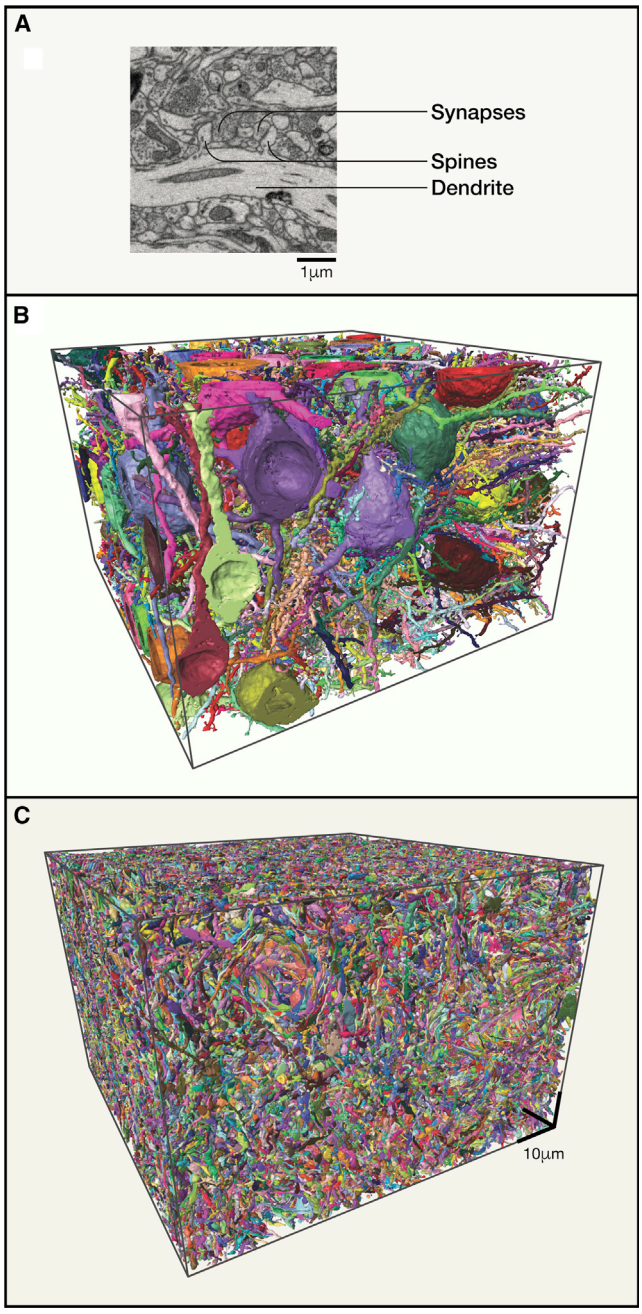

Figure 1. The Complex Synaptic Landscape of the Brain
(A) EM section through mouse somatosensory cortex. A prominent dendrite courses horizontally with spines bearing synapses protruding upward.
(B) EM reconstruction of 89 neurons. Cell bodies and dendrites shown. Each color represents a different neuron.
(C) Reconstruction of 34,221 axonal segments. (B) and (C), same scale (Motta et al., 2019).

strata (laminae) within a target area. This process, called laminar specificity, restricts the choice of partners.

Specificity also occurs at the subcellular level. Many neurons receive synapses from different afferent types on different portions of their surface—for example, distal or proximal dendrites (Figure 2E). Subcellular specificity was initially studied in the neuromuscular system, where regenerating motor axons selectively innervate original synaptic sites on muscle fibers (Sanes et al., 1978; Sanes and Yamagata, 2009), but many cases, some discussed below, have now been analyzed in the central nervous system (CNS).

Finally, some cellular strategies may require limited intercellular recognition, relying instead on coordinated growth of synaptic partners or selective death of potential targets. Although there is some evidence for phenomena of these types (Lopresti et al., 1973; Petrovic and Hummel, 2008; Roberts et al., 2014; Hassan and Hiesinger, 2015; Balaskas et al., 2019), we will not consider them further here.

Progress in understanding mechanisms underlying selective synapse formation has been slow. Comparison to axon guidance highlights the difficulty. Both processes require axons to make many decisions. However, axon guidance can be viewed as a long journey broken into a sequence of choices, many of which are binary and can be analyzed separately. In contrast, synaptic selectivity often requires axons to decide among many alternatives simultaneously. In addition, axon guidance can be evaluated using light microscopy, whereas physiological methods and electron microscopy are required to decide between casual contacts and synapses. An additional obstacle has been that although cell cultures have been effective for analyzing axon growth and synaptogenesis, currently available culture systems do not exhibit robust synaptic specificity.

These differences drove rapid progress in elucidating guidance mechanisms during the 1990s. Although axon guidance is not a solved problem, the major molecular players had been identified by the turn of the century (Tessier-Lavigne and Goodman, 1996; Dickson, 2002). More recently, many mechanisms underlying synaptogenesis have been elucidated (Südhof, 2018). In contrast, only now are tools being developed that allow neurobiologists to tackle synaptic specificity.

New Tools

During the first years of this century, transgenic methods allowed selective marking of specific neuronal types and genome sequence facilitated identification of new candidate recognition molecules. As a consequence of these and other advances, several molecular mediators of partner choice were discovered (Sanes and Yamagata, 2009). Nonetheless, methods were inadequate to provide three types of data required to understand synaptic specificity in a satisfying way: (1) the types of neurons present in a target region, (2) the patterns of synaptic connections between them, and (3) a census of the recognition molecules expressed by synaptic partners. Over the past decade, vastly more powerful methods have been introduced, paving the way for comprehensive characterization of cells (transcriptomes), circuits (connectomes), and molecules (transcriptomes and interactomes).

Cell Types: Transcriptomes

The birth of neurobiology can be traced to Cajal, who spent much of his life classifying neurons using morphological criteria. For nearly a century, morphology remained the principal way in which cell types were defined. Later, morphology was combined

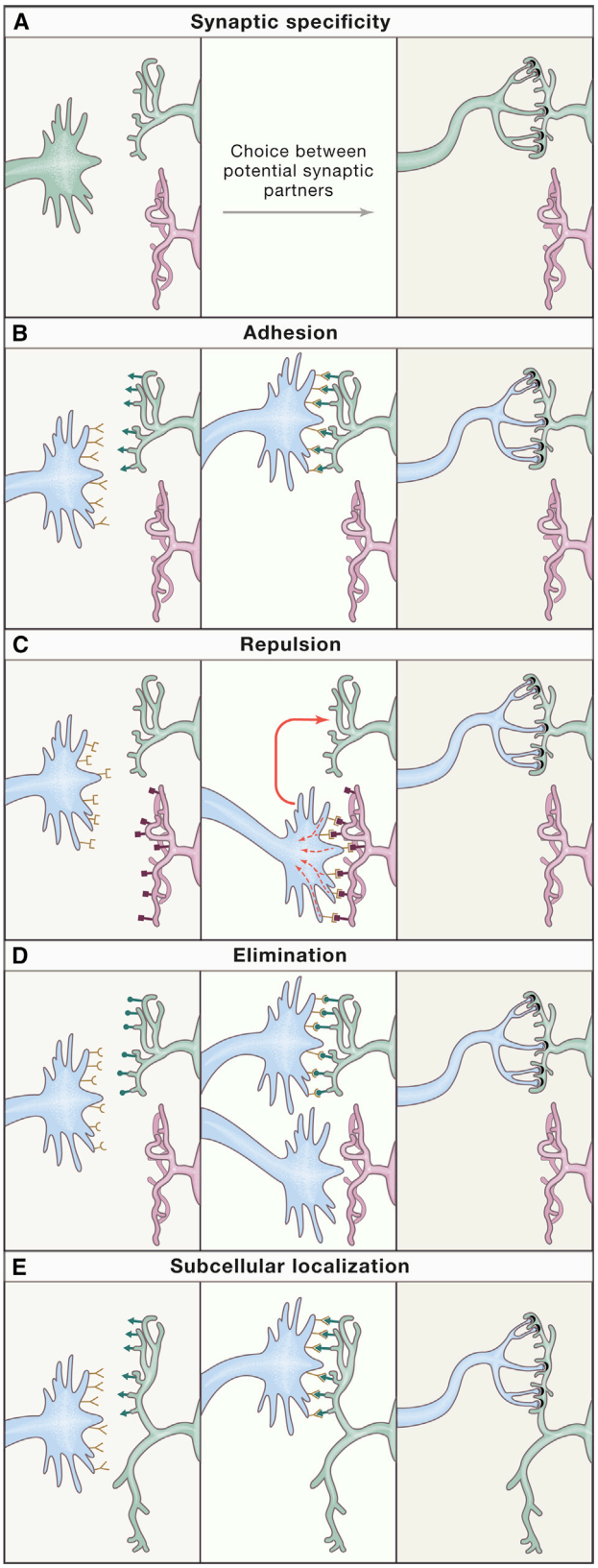

Figure 2. Definition and Potential Mechanisms of Synaptic Specificity

(A) Synaptic specificity: neurites make predictable choices between at least two potential partners.
(B–D) Molecular mechanisms contributing to synaptic specificity.
(B) Preferential adhesion between pre- and postsynaptic partners.
(C) Recognition followed by repulsion, prohibits synapse formation.
(D) Synapses between inappropriate partners are eliminated.
(E) Synapses exhibit subcellular specificity on the postsynaptic cell surface. Subcellular specificity can result from most mechanisms that account for cellular specificity.

with molecular markers, but classification remained incomplete for two reasons: methods were low throughput and markers were obtained serendipitously rather than systematically.

The advent of high throughput single-cell RNA sequencing (scRNA-seq) provided a way to circumvent these limitations. Thousands of cells are collected in a single run and their mRNAs captured by barcoded oligonucleotides; RNA from all cells is then reverse transcribed, amplified, and sequenced together in a single reaction (Figure 4). The barcode allows sequences to be grouped by their cell of origin and computational methods cluster the cells based on transcriptomic similarity. These methods led to huge decreases in time and expense per cell profiled.

Although transcriptomes provide a starting point for classification of cell types, the relationship of cell clusters to cell types is not self-evident. It is possible, however, to match selectively expressed genes to morphology or physiology, and in many cases, the news has been good: 1:1 matches of types defined by molecular, morphological, and physiological criteria are often found (Zeng and Sanes, 2017).

This method was developed independently by three groups (Macosko et al., 2015; Klein et al., 2015; Zheng et al., 2017). Adoption was remarkably rapid, aided by commercialization of one of the systems (https://www.10xgenomics.com). Other methods have been introduced more recently (Kulkarni et al., 2019). Over the past few years, cell atlases of multiple brain regions have been reported. The number of cell types is large—~130 each in mouse retina and visual cortex (Tasic et al., 2018; Yan et al., 2020b). Thus, although some uncertainties remain, we now have, or can readily obtain, "parts lists" of the cells that meet and match in specific target areas.

scRNA-seq can also identify the recognition molecules expressed by specific neuronal types during synapse formation (e.g., Li et al., 2019; Kurmangaliyev et al., 2019). Combined with connectomic analysis, discussed next, candidates expressed by synaptic partners can be identified.

Circuits: Connectomes

Constructing maps of synaptic connectivity, called connectomes, is challenging due to the small size of synapses (generally below the resolution of light microscopy) and the density of neuronal processes in the brain (Figure 1). Progress was long limited by the laborious, low-throughput nature of the two main approaches, electron microscopic (EM) reconstruction and paired electrophysiological stimulation and recording. Over the past decade, connectomics has been revitalized by advances in morphological and physiological methods.

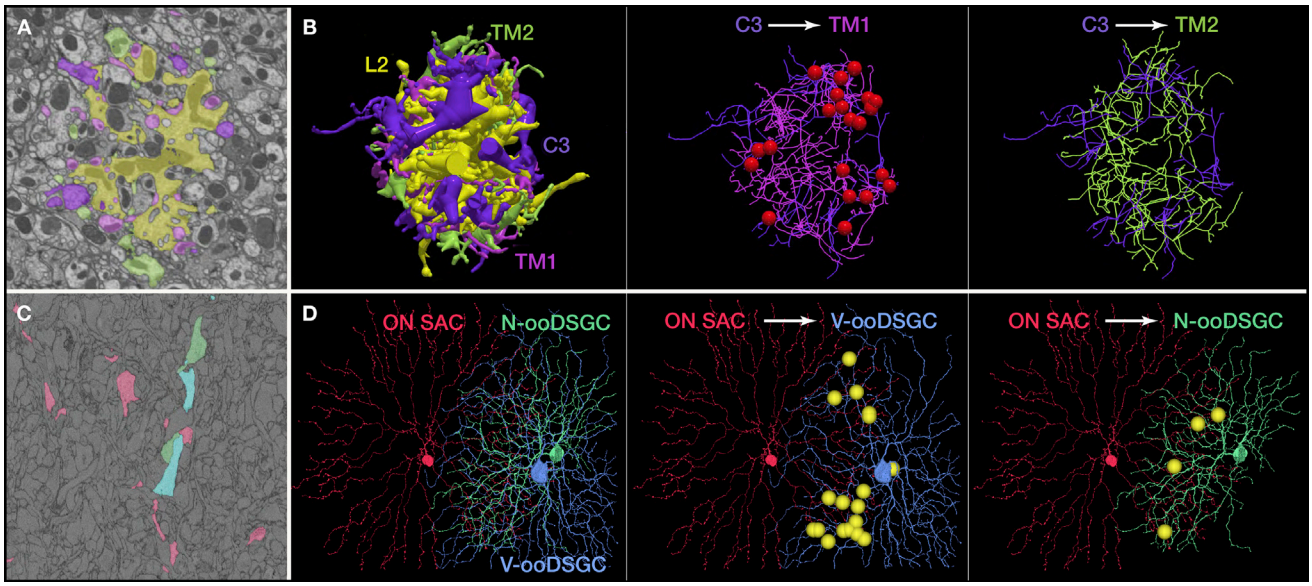

Figure 3. The Extent of Synaptic Specificity Varies from Stringent to Biased

(A) EM section through adult *Drosophila* visual system (see Figure 7). The dendrites of two related neurons, Tm1 and Tm2, are color coded as in (B).

(B) C3 neurons show stringent specificity, forming synapses with Tm1 but not Tm2 (modified from Takemura et al., 2015).

(C) EM section through the IPL (see Figure 8). The processes in color are from starburst amacrine cells (SAC), and two directionally sensitive retinal ganglion cells, called V-ooDSGC and D-ooDSGC.

(D) Synapses form preferentially, in a biased fashion, between the SAC and one RGC type (based on data from Greene et al., 2016; Bae et al., 2018).

Perhaps most impressive have been improvements in EM technology. Each stage in the process has been reimagined, from methods for automated sectioning and imaging to machine learning-based reconstruction. As a result, patterns of synaptic connections have been determined in several regions of the mouse and *Drosophila* CNSs (e.g., Bae et al., 2018; Scheffer and Meinertzhagen, 2019; Motta et al., 2019). In the near term, however, applications of this technology are likely to be limited. First, it is too laborious for assessment of connectivity in multiple specimens so individual variation remains unclear, and it cannot be used for routine analysis of genetic or other perturbations. Second, the reconstructed areas are too small to map long-distance connections. Third, it is challenging to relate morphology to cell type as determined by other criteria. Finally, EM marks synaptic structures but provides no information about their function.

Fortunately, a suite of molecularly based methods are circumventing these limitations. Creative ways to beat the diffraction limit of light microscopy using super-resolution imaging (Sigal et al., 2018) or isotropic expansion (Wassie et al., 2019) enable mapping synaptic connectivity between labeled cells. Advances in viral and transgenic technologies enable tracing of long-distance connections (Luo et al., 2018). Spatial transcriptomic methods provide ways to match molecular signatures to morphology at scale (Lein et al., 2017). Combining optogenetic stimulation of specific cell types with calcium or voltage imaging (“all-optical physiology”) provides a powerful method to assess functional connectivity (Fan et al., 2020). Together, these advances may soon make mapping synaptic connectivity routine.

Binding Partners: Interactomes

Once recognition molecules have been identified, the next important step is to characterize their interactions. To this end, methods have been devised to generate large maps of binding specificity or “interactomes.”

As adhesion molecules frequently have low affinities for each other, their interactions are often difficult to detect in simple binding assays. To address this problem investigators take advantage of avidity, the increased affinity of binding between multimers compared to monomers. Recombinant proteins are generated in which ectodomains are oligomerized, so binding can be detected using ELISA-based assays capable of testing tens of thousands of interactions in a reasonable period of time (Wojtowicz et al., 2007; Bushell et al., 2008) (Figure 5A).

These assays have led to the identification of many interactions among fly and mammalian proteins (Özkan et al., 2013; Visser et al., 2015; Ranaivoson et al., 2019). Along with other methods, such as cell-cell adhesion assays using transfected cells and surface plasmon resonance using recombinant proteins, targeted interactomic efforts have provided insights into binding properties of several families (Figures 5B–5E) (for review, see Honig and Shapiro, 2020). Combined with transcriptomic and connectomic data, interactomes provide a means of predicting mediators of synaptic specificity.

Families of Recognition Molecules Underlie Synaptic Specificity

The search for mechanisms underlying cell-cell interactions has a long history, beginning with embryological experiments in the 1940s and 1950s by Holtfreter, Moscona, and Steinberg

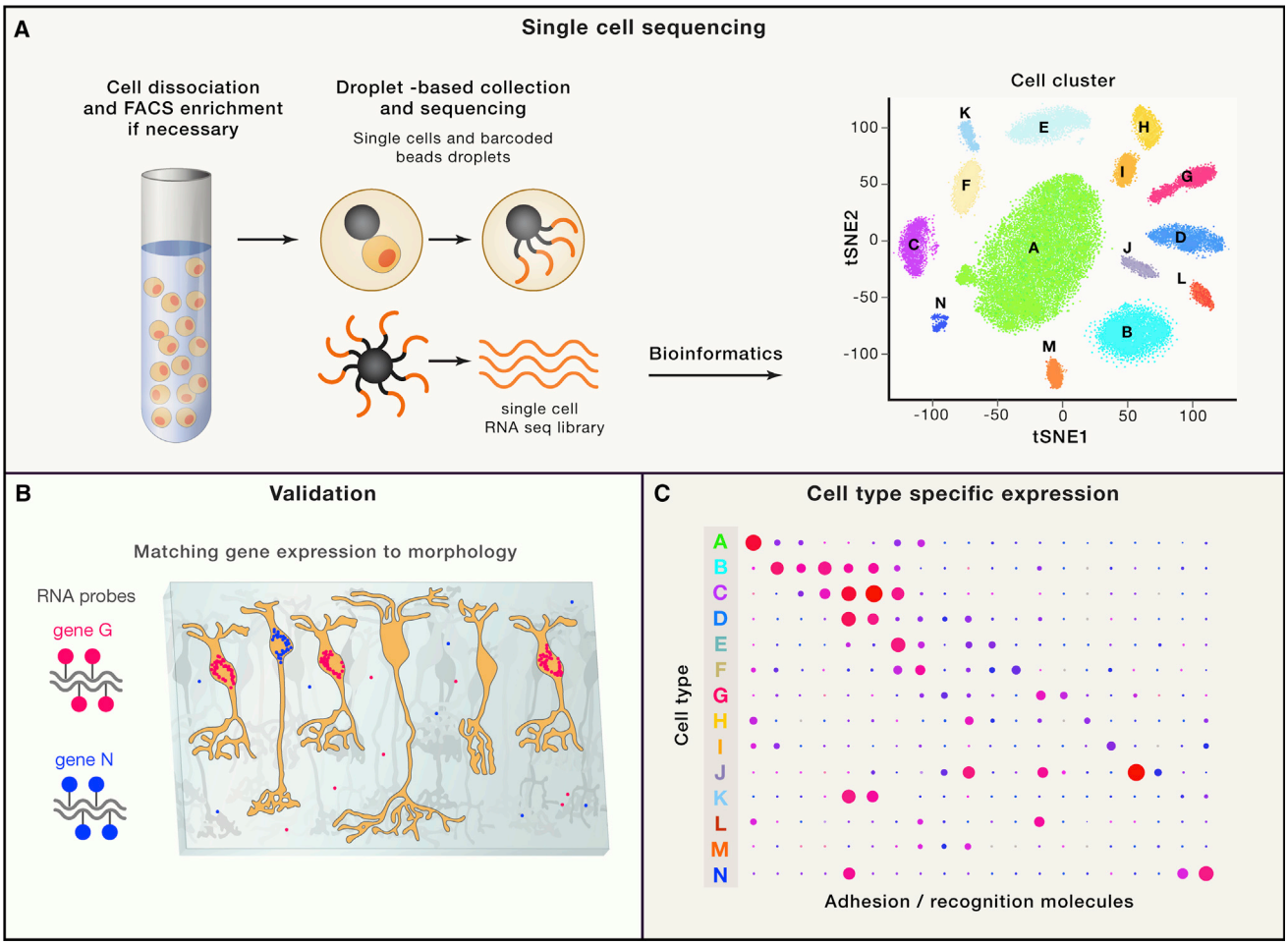

Figure 4. Single-Cell Transcriptomics Classifies Neuronal Types and Reveals Recognition Molecules They Express

(A) Pipeline for high-throughput scRNA-seq. A microfluidic device pairs cells with beads conjugated to barcoded oligo-dT-terminated oligonucleotides in nanoliter-sized oil droplets. Cells lyse, their mRNA is captured, and the mRNA from thousands of cells is reverse transcribed along with the barcode, amplified, and sequenced together. Computational methods cluster transcripts by cell, using the barcodes. Cell clusters are often displayed on t-distributed stochastic neighbor embedding (t-SNE) or UMAP plots, with color and arrangement denoting potential cell types.

(B) Histological methods localize cluster-specific genes to cell types in sparsely labeled tissue, matching molecular identity to morphology and/or physiology.

(C) Data analysis reveals recognition molecules expressed by each cell type.

(Honig and Shapiro, 2020). Their demonstrations that selective affinities among cells drive cell sorting and morphogenesis fueled a search for the molecules that accounted for these processes, but none were found for over two decades. The landscape changed in the 1970s when Takeichi, (1977) demonstrated that cells bear two adhesion systems, calcium-dependent and calcium-independent. Within a few years, his group and others identified the founding members of the calcium-dependent cadherin superfamily, cdh1-3 (Takeichi, 2018). In parallel, Edelman and colleagues (Thiery et al., 1977) described the first calcium-independent adhesion molecule, N-CAM, now known to be a member of the immunoglobulin (Ig) superfamily. These landmark discoveries, along with technical advances, ushered in a new era of discovery (de Wit and Ghosh, 2016). The cadherin and Ig superfamilies have now been joined by other families, such as leucine-rich repeat proteins and teneurins (Figure 6), providing a vast repertoire of specificities that enable neurons to recognize and discriminate among one another.

In this section, we illustrate how these families contribute to synaptic specificity. Given space constraints, we limit our coverage in several respects. First, we do not discuss mediators of axon guidance, synaptogenesis, synaptic plasticity, or synaptic remodeling except when their roles in target recognition are clear. Second, we largely ignore structural studies, because this topic is covered in a companion Review (Honig and Shapiro, 2020). Finally, for those proteins we discuss, we have chosen only a few examples to illustrate how they act, but provide references to their roles in other systems.

Immunoglobulin Superfamily

There are at least 500, 130, and 60 genes encoding Ig superfamily proteins in mice, *Drosophila*, and *C. elegans*, respectively. In each species, at least half are expressed in neurons, and a

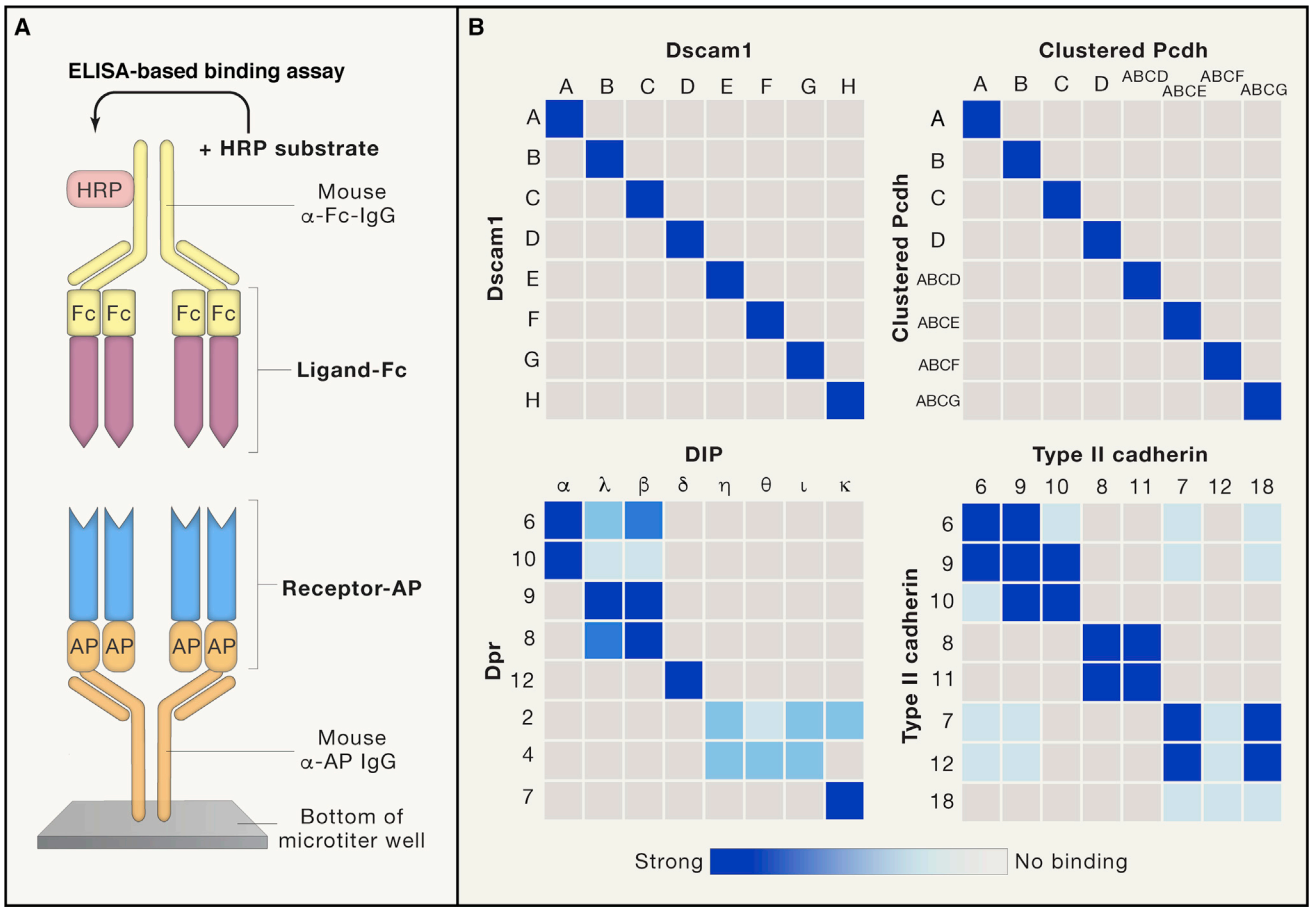

Figure 5. Binding Specificity of Protein Families

(A) ELISA-based screening method (see text).

(B) Binding properties of protein isoforms: clustered Pcdh and Dscam1 proteins show highly specific homophilic binding. Clustered Pcdh proteins form heteroligomers, resulting in additional specificity. DIP/Dpr interactions are largely heterophilic and often promiscuous. Type II cadherins are homophilic, but also exhibit heterophilic binding specificity (data from Shimoyama et al., 2000; Wojtowicz et al., 2007; Thu et al., 2014; Brasch et al., 2018; Cosmanescu et al., 2018).

growing number have been implicated in partner selection. Many of these genes can be further divided into subfamilies. They include Dprs, DIPs, Syg1/Syg2-related proteins, Down syndrome cell adhesion molecules (Dscams), Sidekicks (Sdks), Contactins (Cntns), and L1s. Some of them (e.g., Dscams) bind primarily homophilically, others heterophilically (e.g., Dprs to DIPs), and still others both homophilically and heterophilically (e.g., L1s). Others such as 14 Beats and 8 Sides have been implicated in nerve muscle connectivity; their roles in the CNS are under active investigation (Li et al., 2017).

Vertebrate Dscams, Sdks, and Cntns Mediate Homophilic Recognition

A clade of 14 closely related IgSF members includes most of the vertebrate IgSF genes implicated to date in synaptic specificity: two Sdks, two Dscams and six Cntns discussed here, and four L1s, discussed below. They all contain multiple extracellular Ig domains and fibronectin type III domains (Figure 6A). In Sdks and Dscams, these are followed by transmembrane and cytoplasmic domains terminating in sequences that bind to PDZ domain-containing scaffolding proteins, which can anchor them at synaptic sites (Yamagata and Sanes, 2010; Garrett et al., 2016). Cntns are linked to the membrane by a lipid tail, but generally act as heterodimers with one of six transmembrane Casprs, which terminate in a PDZ-binding sequence.

Several of these proteins have been studied in detail in the vertebrate retina, in which information processing relies on stereotyped patterns of connectivity among >100 types (in mice and chicks) of interneurons and retinal ganglion cells (RGCs) (e.g., Sanes and Masland, 2015; Yan et al., 2020a). Each RGC type is thereby rendered selectively responsive to specific visual features such as motion in a particular direction. During development, processes of these cells intermingle in a narrow inner plexiform layer (IPL), nestled between the inner nuclear and ganglion cell layers, where they recognize specific partners and form circuits (Figure 7A). One feature of this connectivity is that arbors of each neuronal type are confined to one or a few IPL sublaminae. Thus, specificity involves both choices among sublaminae and choices among cell types within sublaminae.

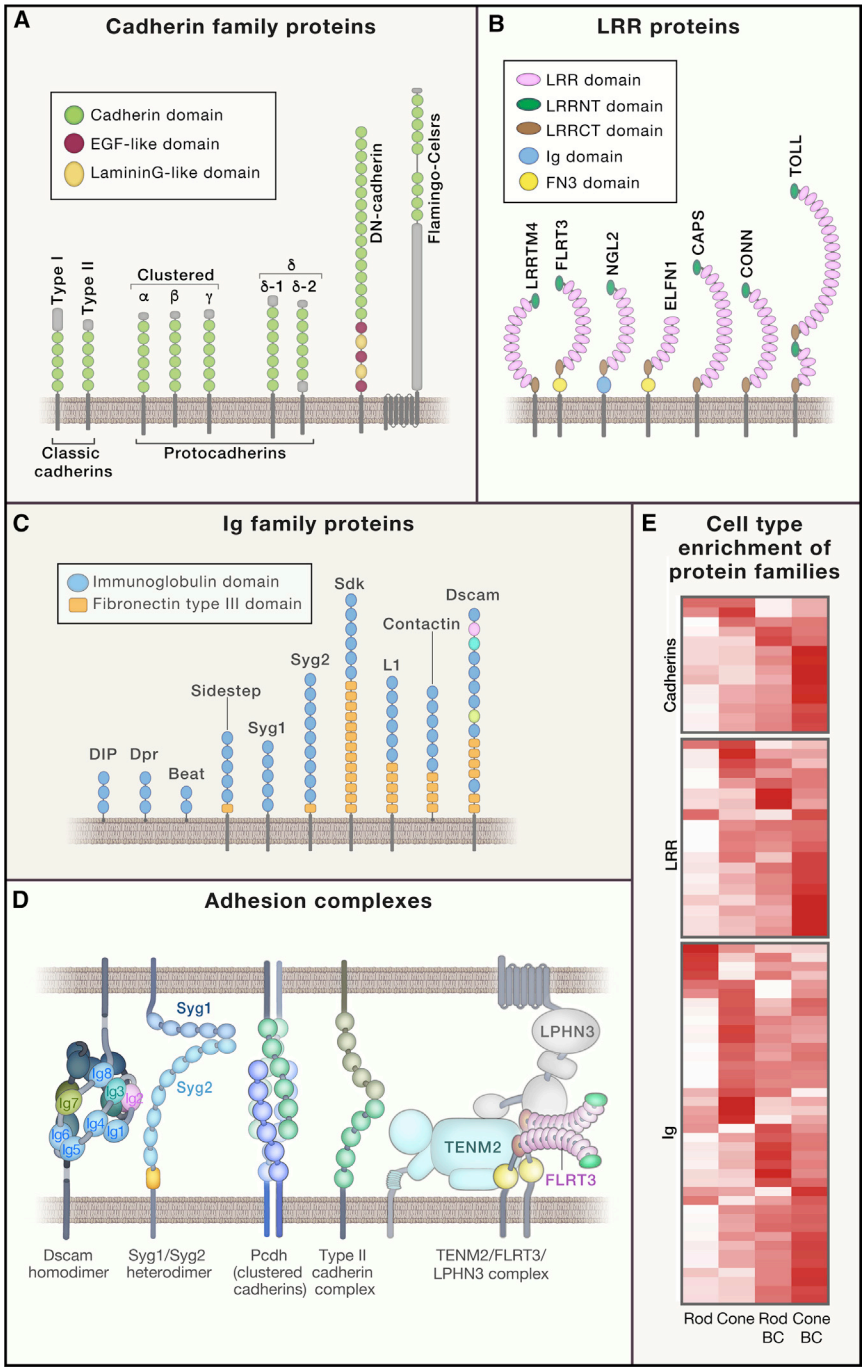

Figure 6. Adhesion Protein Families Regulating Synaptic Specificity

(A–C) Domain structures of protein families comprising cadherin (A), leucine-rich repeat (B), and immunoglobulin (C) domains. Pink, turquoise, and yellow Ig domains represent variable domains in invertebrate Dscam1 proteins.

(D) Structures of adhesion complexes. Dscam1 binding requires precise matching between Ig domains 2, 3, and 7 (Meijers et al., 2007; Sawaya et al., 2008). Syg1 and Syg2 proteins bind through their N-terminal domains (Özkan et al., 2014). Clustered Pcdh proteins form isoform-independent *cis*-dimers through membrane proximal repeats. Homophilic binding specificity occurs through the four N-terminal cadherin repeats unique to each isoform (Rubinstein et al., 2015). Type II cadherins exhibit binding through their N-terminal cadherin domain (Brasch et al., 2018). FLRT3, latrophilin 3, and teneurin 2 form a ternary complex (Li et al., 2020; also see Del Toro et al., 2020).

E. Expression of cell surface proteins in developing synaptic partners. Cadherins, LRR, and Ig superfamily proteins (each row) are differentially expressed by rods, cones, rod bipolars, and cone bipolars (columns) in the mouse retina (Sarin et al., 2018). Red, relative expression with dark red indicating high.

Results obtained in chick suggested that Sdks, Dscams, and Cntns play roles in assembly of these circuits. First, all were expressed in potential pre- and postsynaptic partners. Second, they were expressed in largely non-overlapping neuronal subsets. Third, arbors of neurons that expressed each protein were largely confined to one or a few IPL sublaminae. Finally, all can bind homophilically. Together, these patterns led to the idea that they comprise an ''immunoglobulin superfamily code'' for patterning retinal connectivity. Initial studies using overexpression and knockdown supported this model (Yamagata et al., 2002; Yamagata and Sanes, 2008, 2010, 2012).

Functional analyses were then performed in mouse. Both Sdks are expressed in subsets of mouse retinal neurons that arborize in restricted sublaminae, including an RGC type called W3B and an interneuron type called VG3 (Figure 7B). W3B cells have an unusual property called ''object motion sensing'' in which they respond when the timing of the movement of a small object differs from that of the background, but not when they coincide. This property depends on input from VG3 interneurons. In Sdk2 mutants, the laminar restriction of VG3 and W3B arbors is degraded, there is a selective loss of VG3-W3B synapses, and W3B RGCs are no longer able to distinguish global from local motion (Krishnaswamy et al., 2015). Sdk1 may act similarly in distinct sets of interneurons and RGCs (Yamagata and Sanes, 2019).

Expression and roles of Dscams in mouse retina are similar in some respects to those of Sdks: they are expressed in neuronal subsets, and mutants exhibit defects in laminar restriction of processes and circuits (Fuerst et al., 2008, 2009, 2012; Li et al., 2015; Simmons et al., 2017). However, Dscams, unlike Sdks, appear to function by preventing interactions among neurons of the same type. This self-avoidance does not reflect

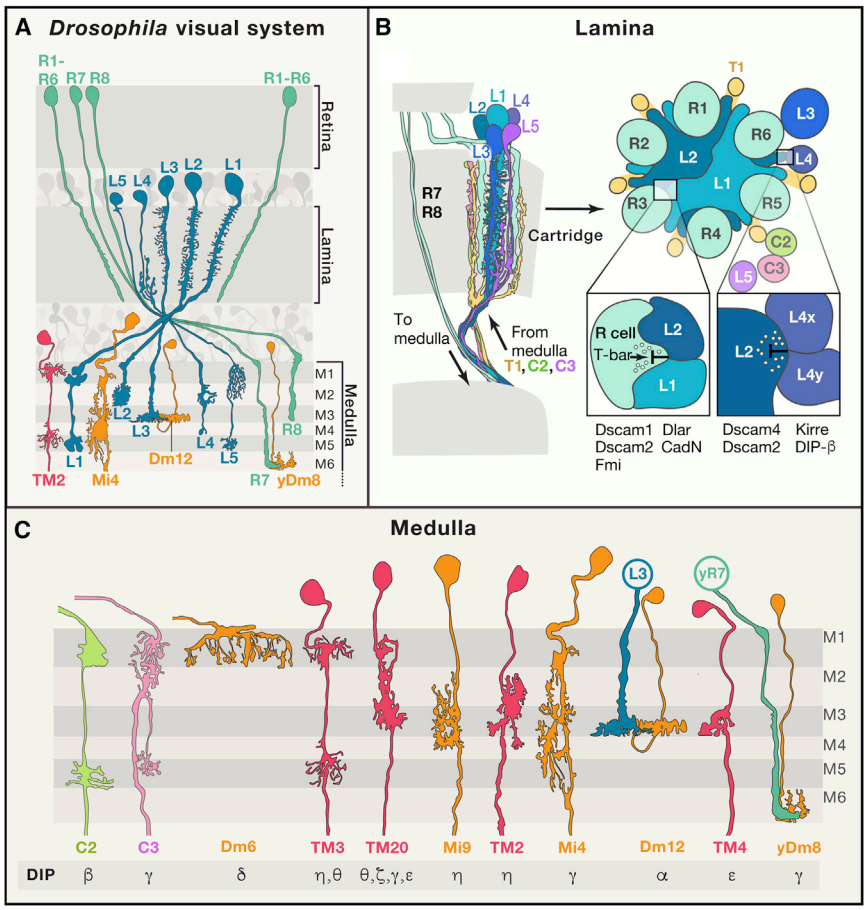

Figure 7. Synaptic Specificity in the *Drosophila* Visual System

(A) Schematic of retina, lamina, and medulla. The processes of >100 different neuronal cell types form synapses in the lamina and medulla. These include different types of photoreceptor neurons (R cells), transmedullary (Tm) neurons, medulla intrinsic (Mi) neurons, and amacrine-like Distal medulla (Dm) neurons.

(B) Combinations of cell surface proteins regulating synapse formation in the lamina are shown for synapses between R cell presynaptic terminals and L1 and L2 postsynaptic sites and for connections between L2 and L4. L2 is presynaptic to dendrites of different L4 neurons (L4y and L4x). The T-bar is an ultrastructural feature of presynaptic active zones.

(C) DIP/Dpr proteins are expressed in medulla neurons (Tan et al., 2015; Cosmanescu et al., 2018). Developing neurons express many Dprs in a dynamic fashion but one or a few DIPs. DIP-α/Dpr6, Dpr10, and DIP-γ/Dpr11 ligand receptor pairs regulate interactions between synaptic partners L3/Dm12 and yR7/yDm8, respectively.

repulsive interactions of the sort employed by other recognition molecules discussed below. Rather, homophilic interactions appear to mask cell-type-specific recognition systems (Garrett et al., 2018). Cntn5 acts in yet a third way: it is expressed selectively by an interneuronal and an RGC type, and acts homophilically to promote connections between the two. Here, the homophilic interaction is required to stabilize synapses between interneuronal and RGC dendrites (Peng et al., 2017).

Thus, although loss of Sdks, Dscams, and Cntns have superficially similar effects on retinal circuitry, they act in different ways, including adhesive matching of synaptic partners, sublaminar restrictions, masking of adhesive interactions, and regulating dendritic morphogenesis. These findings highlight the general point that while structure underlies function it does not necessarily predict it.

The Vertebrate L1 Protein Family Mediates Subcellular Specificity

The L1 subfamily of IgSF recognition molecules comprises four members: L1 (also called Ng-CAM), close homolog of L1 (CHL1), neuron-glia-related cell adhesion molecule (NrCAM), and neurofascin (Nfasc). All four underlie subcellular specificity in GABAergic neurons of the rodent brain.

Nfasc helps confine synapses of GABAergic basket cell interneurons to the axon hillock of Purkinje cells in the cerebellum (Figure 8A). Nfasc is distributed in a gradient on the Purkinje cell surface with levels highest at the axon hillock and lower across the somata and dendrites. Basket cell axons initially innervate the somata and proximal dendrites and then appear to climb the gradient to reach their definitive synaptic sites in a process that also requires Sema3A and its receptor Neuropilin 1 (Ango et al., 2004; Telley et al., 2016). More recently, a similar gradient mechanism requiring L1 was demonstrated in cortical pyramidal neurons (Tai et al., 2019). An additional similarity is that in both cases, cytoskeletal ankyrin-G is needed to localize L1.

The other two family members appear to play related roles: CHL1 and NrCAM enable a specific class of GABAergic interneurons in spinal cord to synapse on axon terminals of primary sensory neurons (Ashrafi et al., 2014; Betley et al., 2009) and CHL1 in cerebellum guides stellate interneuron axons along Bergmann glial processes to appropriate sites on Purkinje cell dendrites (Ango et al., 2008).

Fly Dips and Dprs Match Synaptic Partners through Heterophilic Recognition

A set of 21 Dpr and 11 DIP *Drosophila* Ig superfamily proteins contain two and three Ig domains, respectively. Some bind heterophilically to one partner, others are more promiscuous, and a few bind homophilically (Özkan et al., 2013; Carrillo et al., 2015; Cosmanescu et al., 2018). They exhibit striking cell-type-specific expression, with matched DIP/Dpr cognate proteins often expressed in synaptic partners and sometimes concentrated at synapses (Carrillo et al., 2015; Tan et al., 2015; Xu et al., 2018, 2019). DIPs and Dprs regulate wiring in several different developmental contexts (Barish et al., 2018; Ashley et al., 2019; Venkatasubramanian et al., 2019; Carrillo et al., 2015; Xu et al., 2018; Courgeon and Desplan, 2019). Here, we focus on the developing visual system (Figure 9).

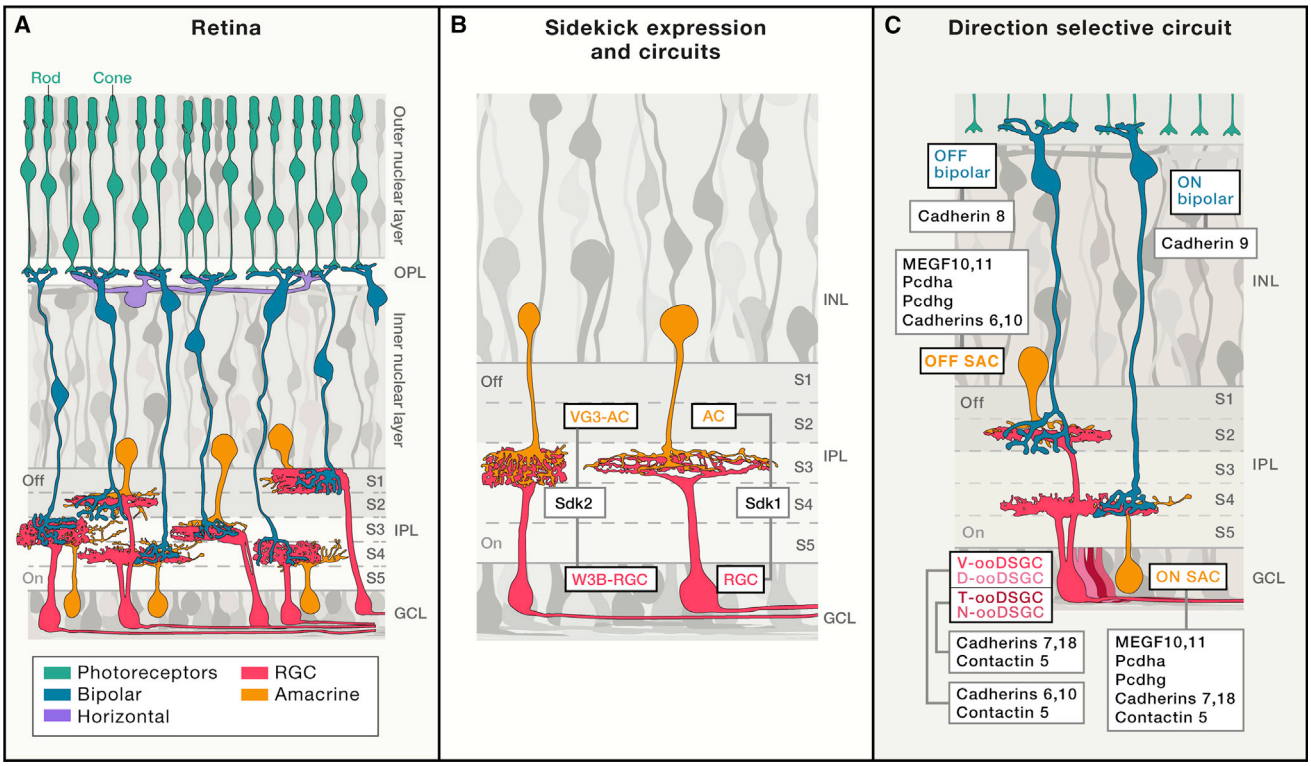

Figure 8. Synaptic Specificity in the Mouse Retina

(A) Basic plan of the retina is conserved among vertebrates. Photoreceptors (rods and cones), interneurons (horizontal, bipolar, and amacrine cells), and retinal ganglion cells (RGCs) are shown. Photoreceptors synapse on horizontal and bipolar cells in an outer synaptic (plexiform) layer; bipolar and amacrine cells synapse on each other and on RGCs cells in an inner plexiform layer (IPL). Axons of RGCs extend through the optic nerve to the brain. The five neuronal classes are divided into ~130 types in mouse. The IPL is divided into sublaminae (shown as S1–S5) with processes of each neuronal type confined to one or a few of them.

(B) Sidekicks (Sdks) 1 and 2 are selectively expressed by pairs of amacrine cells and RGCs that form synapses in S3. Deletion of Sdk2 disrupts connectivity between Sdk2-expressing cells without affecting synapses of either cell type with other partners (Krishnaswamy et al., 2015; Yamagata and Sanes, 2019)

(C) Four types of ON-OFF direction-selective RGCs (ooDSGCs) respond preferentially to motion in one of the four cardinal directions. Their bistratified dendrites receive synapses from ON bipolar and starburst amacrine cells in S4 of the IPL, and from OFF bipolars and starbursts in S2. Multiple recognition molecules expressed by these cell types interact to assemble the circuit. Some, but not all, are described in the text (Kay et al., 2011; Lefebvre et al., 2012; Duan et al., 2014, 2018; Peng et al., 2017; Ing-Esteves et al., 2018).

DIP-α and its binding partners, Dpr 6 and Dpr10, regulate connectivity of neurons called Dm12 in the medulla region of the visual system (Xu et al., 2018). Dm12 expresses DIP-α and its synaptic partner L3 expresses both Dpr6 and Dpr10. Binding between DIP-α and Dpr6/10 regulates both target specificity and synapse number. Selective removal of DIP-α from single Dm12 neurons, or selective manipulation of amino acid sequences abrogating DIP-α interactions with Dpr6/10, lead to mistargeting and elaboration of synapses with inappropriate partners and to reduction in the number of "appropriate" synapses. The interaction is instructive, as shown by the finding that altering the layer distribution of Dpr6 or Dpr10 redirects Dm12 axons to target to this layer.

DIP/Dpr proteins also mediate other interactions in the visual system. For example, loss of function mutations in DIP-γ and Dpr11 lead to alterations in morphology of neurites of a subtype of R7 neurons, yR7, and its synaptic partner, yDm8 (Carrillo et al., 2015; Menon et al., 2019) and DIP-β regulates fidelity of synapses between L4 and L2 (Xu et al., 2019). DIP/Dpr interactions also regulate partner choice by affecting apoptosis of specific neuronal types prior to synapse formation (Carrillo et al., 2015; Courgeon and Desplan, 2019; Xu et al., 2018). However, synaptic pairs expressing other matched DIP/Dpr proteins do not show obvious mutant phenotypes (Xu et al., 2018), and the defects described above are only partially penetrant. Thus, during normal development the presence of matched DIP/Dpr pairs on neurons is not sufficient to determine specificity, so other recognition molecules also must contribute to this process.

DIP and Dpr proteins are related to RIG-5 and ZIG-8 in *C. elegans*, and the IgLON family comprising five proteins in mammals. These have not been studied in the context of synaptic specificity but RIG-5 and ZIG-8 regulate adhesion between neurons (Bénard et al., 2012), and IgLONs are localized to synapses and can control synapse number *in vitro* (Cheng et al., 2019).

C. elegans Syg1 and Syg2 Mediate Interactions with Temporary Targets

In some cases, neurons form transient synapses prior to forming definitive synapses. In *C. elegans*, for example, two Ig superfamily transmembrane proteins, Syg1 and Syg2, which bind to each

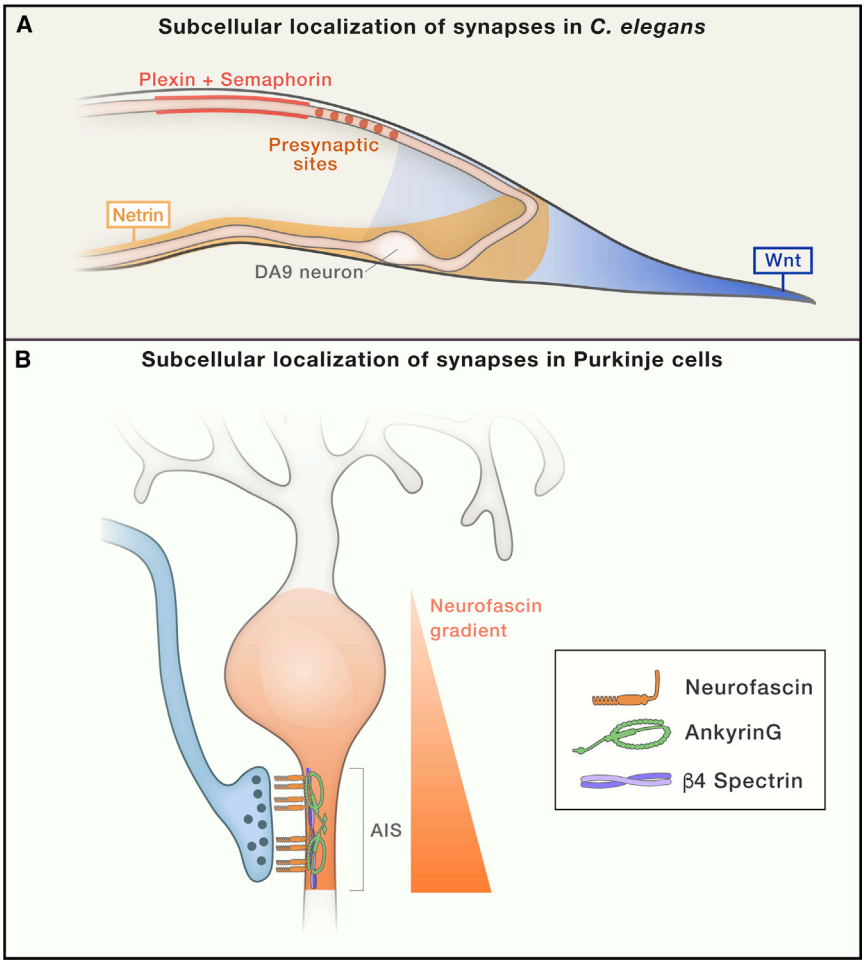

Figure 9. Subcellular Specificity
(A) Three signaling pathways, involving Wnt, Netrin, and Plexin/Semaphorin, regulate the precise distribution of presynaptic sites.
(B) A gradient of Neurofascin proteins supported by localized ankyrin and spectrin on the inner face of the plasma membrane promotes targeting of inhibitory basket axons to the axon initial segment.

other, regulate synapses made by a motor neuron called HSNL (Shen and Bargmann, 2003; Shen et al., 2004). When HSNL axons arrive in their target region, they synapse transiently on epidermal cells before forming definitive synapses on muscle cells and neurons. The transient synapse requires binding of Syg1, expressed by HSNL, to Syg2, expressed by the epidermal cells. In the absence of these proteins, HSNL forms synapses at ectopic locations. The interaction nucleates the assembly of a presynaptic structure in HSNL axons at a precise location defined by the position of Syg2. In addition, Syg1 inhibits an E3 ubiquitin ligase that targets degradation of presynaptic sites elsewhere in the axon (Ding et al., 2007). Thus, precise spatial localization of synapses relies upon targeted assembly of the presynaptic sites and inhibition of a broadly acting degradation machinery in the axon.

C. elegans Syg proteins have relatives in *Drosophila* and mammals (Syg1: Rst and Kirre/Duf [*Drosophila*] and Neph1-3/Kirrel1-3 [mouse]; Syg2: SNS and Hbs [*Drosophila*], nephrin [mouse]). These are also involved in synaptic specificity. For example, mouse Kirrels mediate synaptic selectivity of olfactory receptor axons in the olfactory bulb (Serizawa et al., 2006; Vaddadi et al., 2019), and *Drosophila* kirre mediates synapse formation in the fly visual system (Lüthy et al., 2014).

Fly Dscams Pattern Synapses through Self-Avoidance and Dendritic Targeting

There are four Dscam genes in *Drosophila*. Dscam1 was identified through biochemical and genetic studies while Dscam2–Dscam4 were identified by homology. Dscam2–Dscam4 are conventional recognition molecules, but Dscam1 exhibits astonishing diversity. Alternative RNA splicing results in the production of >18,000 Dscam1 isoforms that differ at sites within three Ig domains (Schmucker et al., 2000; Wojtowicz, 2004, 2007; Sun et al., 2013b). Each ectodomain exhibits isoform-specific homophilic recognition (Figure 5B). Isoforms comprising the same three variable domains bind to each other whereas isoforms differing by any one of the three do not.

The extraordinary multiplicity of isoforms and their exquisite binding specificity immediately suggested roles for Dscam1 in neural circuit assembly. In its simplest form, identical isoforms expressed on synaptic partners would contribute to synaptic specificity through homophilic recognition. To date, however, there is no compelling evidence for this mechanism (Chen et al., 2006; Hattori et al., 2009). Instead, binding activates repulsion, leading to self-avoidance, the tendency of processes of the same cell to be selectively repelled from one another (Zhan et al., 2004; Matthews et al., 2007; Soba et al., 2007; Hughes et al., 2007).

The multiplicity of isoforms is essential for neurons to discriminate between self and non-self (Hattori et al., 2007, 2009). Neurons express ~10–40 Dscam isoforms in a probabilistic way, thereby endowing each individual neuron with a unique identity (Neves et al., 2004; Zhan et al., 2004; Miura et al., 2013). This pattern allows neurites of a single neuron to avoid each other, while being "blind" to other neurons of the same type. Gain-of-function and loss-of-function studies showed that neurons must express thousands of isoforms for normal patterning of axons, dendrites and synapses, but the specific isoforms they express are unimportant; it is only important that the isoforms expressed by one neuron are different from those of its neighbors (Wang et al., 2004; Zhan et al., 2004; Chen et al., 2006; He et al., 2014; Hattori et al., 2007, 2009; Wu et al., 2012).

Dscam2 and Dscam4 also exhibit homophilic binding and contribute to synaptic specificity. Dscam2 acts alone and in

combination with Dscam1 to pattern synapses of L1 and L2 neurons by repulsive interactions (Millard et al., 2007, 2010; Kerwin et al., 2018). In contrast, Dscam2 and Dscam4 act by an adhesive mechanism in L4 neurons and their normal targets; L4 forms synapses on additional targets in Dscam2/4 double mutants (Tadros et al., 2016). Dscam1 also interacts with other ligands and receptors to regulate branching and axon guidance in the developing CNS (Andrews et al., 2008; Alavi et al., 2016; Dascenco et al., 2015).

Cadherin Superfamily

The cadherin superfamily contains >100 members in mammals, 19 in flies, and 13 in *C. elegans*. Its founding members (vertebrate Cdh1–Cdh3) and ~15 of their closest relatives are called classical cadherins. They are homophilic adhesion molecules with a conserved cytoplasmic domain that binds signal transducing proteins called catenins (Hirano and Takeichi, 2012). Other subfamilies, discovered later, include clustered and delta protocadherins (Pcdhs).

Vertebrate Type II Cadherins Regulate Assembly of a Retinal Direction-Selective Circuit

The classical cadherins are divided into types I (Cdh1–Cdh4 and Cdh15) and II (Cdh5–Cdh12, Cdh18–Cdh20, Cdh22, and Cdh24). They are expressed in combinatorial patterns throughout the CNS and have been implicated in several aspects of neural development.

Type II cadherins regulate circuit assembly in hippocampus, spinal cord, cerebellum, and retina (Basu et al., 2015, 2017; Williams et al., 2011; Kuwako et al., 2014; Suzuki et al., 2007; Liu et al., 2018). In retina, on which we focus here, the circuit is one that reports the direction in which objects are moving (Figure 8C). It comprises eight cell types. The RGCs are called ON-OFF direction-selective ganglion cells (ooDSGCs) because they respond to moving objects that are either brighter (ON) or darker than the background (OFF). Each of four ooDSGC types is tuned to motion along a cardinal axis—ventral, dorsal, nasal, or temporal—and is molecularly distinct (Kay et al., 2011). Sets of ON and OFF bipolar interneurons, (primarily types BC5 and BC2, respectively) receive synapses from photoreceptors, and then deliver ON and OFF input to all four types of ooDSGCs, as well as to ON and OFF starburst amacrine cells. The starbursts, in turn form directionally tuned inhibitory synapses on the ooDSGCs (Figure 3B), which renders them directionally selective (Wei, 2018).

At least 15 classical cadherins are expressed by one or more of these cell types and at least six (Cdh6, Cdh7, Chd8, Cdh9, Cdh10, and Cdh18) act to specify the synapses they form (Duan et al., 2014, 2018). Cdh8 targets BC2 axons to dendrites of ooDSGCs and OFF starbursts; Cdh9 targets BC5 axons to dendrites of ooDSGCs and ON starbursts; Cdh6, Cdh9, and Cdh10 target starbursts to dendrites of dorsal- and ventral-preferring ooDSGC; and Cdh7 and Cdh18 target starbursts to dendrites of nasal and possibly temporal-preferring ooDSGC. This division of labor is reflected in the physiological phenotypes of Cdh mutants; for example, OFF responses of ooDSGCs are decreased in Cdh8 mutants, leaving ON responses intact and direction-selective, whereas deletion of Cdh6, Cdh9, and Cdh10 (but not of any single Cdh) renders dorsal and ventral ooDSGCs responsive to flashes of light but largely direction-non-selective. Thus, each Cdh or set of Cdhs specifies a unique synaptic type that subserves a unique function within a complex circuit.

Fly DNCad: A Single Cadherin Regulates Targeting in Many Different Neurons

In contrast to the mammalian retina, in which multiple classical cadherins regulate specificity, a single classical cadherin, CadN, has been implicated in *Drosophila* (Iwai et al., 1997, 2002; Lee et al., 2001; Hummel and Zipursky, 2004; Prakash et al., 2005). It plays multiple roles, likely reflecting dynamic regulation of its level and subcellular distribution, and interactions with other recognition molecules. Here, we consider its role in the visual system.

Each of ~700 cartridges in the lamina region is a synaptic unit that processes visual information from a specific point in the visual field. A cartridge comprises portions of the axons and dendrites of six photoreceptor cells (R1–R6), five lamina monopolar neurons (L1–L5), and several other neuron types. L1 and L2 lie in the center of the cartridge, surrounded by R1–R6 axons; these, in turn, are surrounded by processes of L3–L5. This arrangement is elaborated through initial interactions between R cell growth cones and later between these growth cones and lamina neurons (Clandinin and Zipursky, 2000; Langen et al., 2015).

The position of axons and dendrites within the cartridge is determined by the relative levels of CadN. High levels of CadN place L1 and L2 neurons within the center of the cartridge, lower levels place R cell terminals around L1 and L2, and even lower levels in L3–L5 place these neurons outside R cell axons. Changing the levels of CadN alters the position of axons and dendrites within the cartridge. Thus, the onion-like concentric arrangement of processes results from differences in the level of adhesivity, providing *in vivo* support for the differential adhesion model of Steinberg in which the organization of cells is determined by different levels of a single adhesion protein. Removing CadN selectively from either lamina neurons or R cells leads to an ~80% reduction of synapses between them (Schwabe et al., 2013, 2014).

Additional functions of CadN arise from its interaction with other cell surface proteins. Targeting of R1–R6 cells occurs in two steps with CadN playing distinct roles in each. First, Flamingo (Fmi), a divergent cadherin, acts with CadN in R cell terminals, promoting the selection of appropriate postsynaptic targets (Lee et al., 2003; Chen and Clandinin, 2008; Schwabe et al., 2013). In a second step, CadN promotes adhesion between synaptic partners in combination with the receptor protein tyrosine phosphatase Lar (Clandinin et al., 2001). CadN also acts in combination with members of the plexin and semaphorin families to regulate synaptic target layer specificity (Nern et al., 2008; Pecot et al., 2014). Thus, CadN acts in combination with different cell surface proteins to promote a range of interactions between processes in the developing target region.

Clustered Protocadherins Regulate Self-Avoidance in the Mammalian CNS

Protocadherins (Pcdh) comprise several groups with extracellular domains similar to those in classical cadherins, but divergent cytoplasmic domains. The largest set, the clustered Pcdhs, is encoded by a tandem array of genes at a single genomic locus. They are subdivided into three groups, α, β, and γ, with 14, 22, and 22 isoforms, respectively, in mice (Kohmura et al., 1998;

Wu and Maniatis, 1999; Mountoufaris et al., 2018). In the α and γ clusters, each ectodomain, transmembrane domain, and a short juxtamembranous stub is encoded by a single exon, which in each case is transcribed from its own promoter and spliced to three common exons that encode a shared (constant) cytoplasmic domain. Each single β cluster exon encodes a complete protein with a short cytoplasmic domain.

Although the clustered Pcdhs are structurally unrelated to fly Dscam1 described above, they are remarkably similar in many other respects. First, most isoforms engage in strict homophilic binding (Schreiner and Weiner, 2010; Thu et al., 2014). This pattern might suggest the existence of 58 binding specificities, but in fact the number is vastly greater, because isoforms from the same and different clusters multimerize promiscuously in *cis*, with efficient *trans*-binding requiring a near-perfect match of monomeric components (Rubinstein et al., 2015). Second, although diversity arises from promoter choice in Pcdhs and alternative splicing in Dscam1, the result in both cases is that a vast array of extracellular binding moieties activates a single intracellular signal transduction cascade. Third, clustered Pcdhs, like Dscam1 isoforms, are expressed in a combinatorial and stochastic fashion in at least some populations (Esumi et al., 2005; Kaneko et al., 2006; Hirano et al., 2012; Toyoda et al., 2014; Mountoufaris et al., 2018). Fourth, clustered Pcdhs mediate self-avoidance in several neuronal populations, including dendrites of retinal starburst amacrine cells and cerebellar Purkinje cells and axons of olfactory sensory and serotonergic neurons; they also mediate self-/non-self-discrimination in at least starburst amacrine cells (Lefebvre et al., 2012; Meguro et al., 2015; Mountoufaris et al., 2017; Chen et al., 2017; Katori et al., 2017; Ing-Esteves et al., 2018). Although specific isoforms play unique roles in some cases (Chen et al., 2012, 2017; Katori et al., 2017), in general, as with Dscam1, the specific isoforms expressed in a neuron are unimportant, it is only important that they are different from one another.

The physiological significance of self-avoidance and self-/non-self-discrimination has been demonstrated in the retina's direction-selective circuit (Kostadinov and Sanes 2015). Dendrites of many starburst amacrine cells form inhibitory synapses on each other, which enhances the directional selectivity of ooDSGCs. Thus, for optimal function, starburst dendrites must avoid forming synapses on sibling dendrites (autapses), while forming synapses on dendrites of other starbursts, which are nominally identical except for expression of different Pcdh isoforms. Starbursts in Pcdhγ null mice form autapses, as if they mistake their own dendrites for dendrites of other starbursts. Conversely, when only a single isoform is expressed in all starbursts, they form few synapses with each other, as if they mistake dendrites of other starburst for their own dendrites. In both cases, directional selectivity of the ooDSGCs is degraded. Thus, self-/non-self-discrimination contributes to synaptic specificity in these neurons.

Leucine-Rich Repeat Family

The leucine-rich repeat (LRR) superfamily is named for a 24-amino acid domain found in both intracellular and extracellular proteins. Leucine-rich repeats form a characteristic binding interface promoting a broad spectrum of interactions.

Several LRRs regulate synaptic target specificity in *Drosophila*. They include Toll proteins (9 paralogs), capricious (2 paralogs), connectin (Kurusu et al., 2008; Nose et al., 1992, 1997; Shishido et al., 1998), and Fili (Xie et al., 2019). For example, Toll regulates targeting of motoneuron axons to innervate appropriate muscles. Here, Toll/Toll homophilic interactions appear to be repulsive in nature; loss of function mutations result in innervation of incorrect targets (Halfon et al., 1995; Rose et al., 1997). Toll 6 and Toll 7 regulate the targeting of different subclasses of olfactory receptor neurons to their appropriate projection neuron targets (Ward et al., 2015) and Fili is required in projection neurons (PNs) to prevent inappropriate targeting of axons to specific glomeruli (Xie et al., 2019) (Figure 10). Capricious patterns projection neuron dendrites in the glomeruli of the antennal (olfactory) lobe and also regulates targeting of R8 growth cones in the visual system (Shinza-Kameda et al., 2006; Hong et al., 2009).

Several mammalian LRR proteins have also been implicated in synaptic specificity, although in most cases it remains unclear whether they promote specificity per se or synaptogenesis. They include three mammalian FLRTs, four LRRTMs, six Slitrks, five SALMs, and two NGLs1. Most bind heterophilically, including FLRTs to latrophilins (Figure 5; see below), LRRTMs to neurexins and LAR receptor tyrosine phosphatases, and NGLs to netrin G1 and G2 (for review, see Südhof, 2018).

Teneurins

Teneurins (Tens) are large type II transmembrane proteins, initially discovered in *Drosophila* (Baumgartner and Chiquet-Ehrismann, 1993; Tucker, 2018) and later shown to regulate connectivity in flies and mice. There are two *Drosophila* and four mammalian teneurins. They are differentially expressed by neuronal types and differentially distributed within single neurons.

Mammalian Teneurins bind both homophilically and heterophilically to latrophilins in the hippocampus (Rubin et al., 2002; Silva et al., 2011). Homophilic interactions of Ten-3 regulate targeting of proximal CA1 neurons to the distal subiculum (Berns et al., 2018), whereas heterophilic interactions of Ten-2 with Latrophilins 2 and 3 pattern inputs to CA1. Lphn2 and Lphn3 are expressed in dendritic regions receiving input from the entorhinal cortex and Schaeffer collaterals, respectively. Lphn3 binds simultaneously to both Ten-2 and Flrt3 on Schaeffer collaterals to promote synapse formation (Sando et al., 2019).

The two *Drosophila* Teneurins, Ten-a and Tem-m, also regulate synaptic interactions (DePew et al.,2019). In the motor system, Ten proteins act heterophilically to promote synapse development and Ten-m acts in a subset of synapses to regulate targeting specificity via homophilic interactions (Mosca et al., 2012). In the olfactory system, Ten-a and Ten-m are expressed in olfactory sensory neurons and their postsynaptic targets, and gain- and loss-of-function studies are consistent with them regulating synaptic specificity by a homophilic mechanism (Hong et al., 2012) (Figure 10).

Axon Guidance Molecules

Several proteins initially studied as axon guidance cues and, in some cases, regulators of embryonic patterning were subsequently implicated in synaptic specificity. Here, we describe roles of several of them in specificity.

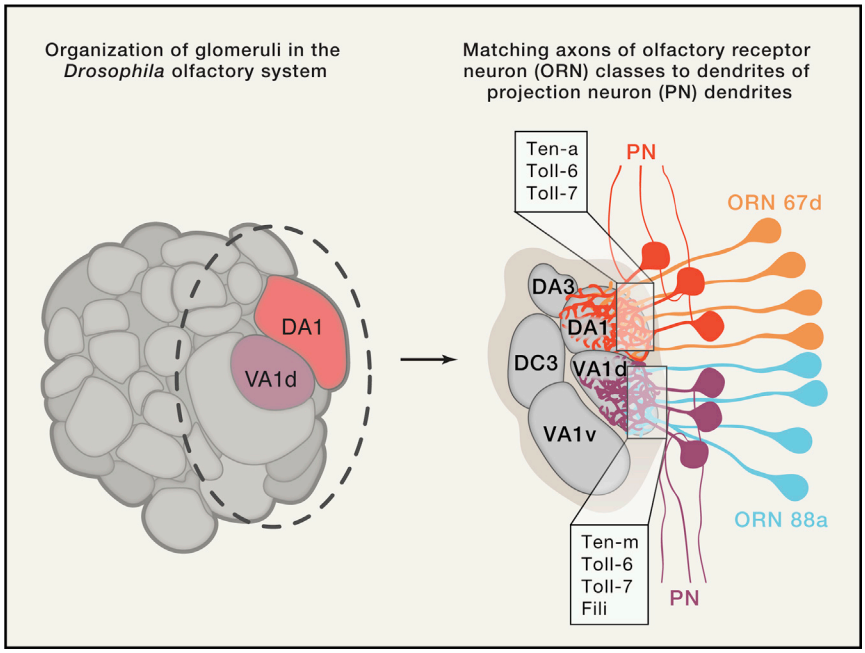

Figure 10. LRR and Teneurins Regulate Synaptic Specificity in the *Drosophila* Olfactory System

The antennal lobe of the fly olfactory system comprises synaptic units called glomeruli, which contain synapses between matched olfactory receptor neurons (ORNs) and dendrites of projection neurons (PNs). Different combinations of teneurins (Ten-a and Ten-m) and leucine-rich repeat proteins (Toll-6, Toll-7, and Fili) regulate targeting to the VA1d and DA1 glomeruli. Some act in either PNs or ORNs, others in both, and these act through adhesion and repulsion.

Repellent Effects of Semaphorins and Plexins Pattern Connections

Semaphorins were identified as repellent axon guidance cues in vertebrates (Luo et al., 1993, 1995) and insects (Kolodkin et al., 1993). There are ~20 semaphorins in mammals (Alto and Terman, 2017). Some are secreted proteins, capable of acting in soluble form while others are transmembrane proteins that mediate cell-cell interactions. All bind to plexins, a group of nine related proteins in mice that are themselves distant relatives of the semaphorins. Some semaphorins signal through plexins alone while others, particularly secreted semaphorins, signal through complexes of plexin and one of two neuropilins. The *Drosophila* genome encodes two secreted semaphorins, two transmembrane semaphorins, and two plexins but no neuropilins.

Semaphorins and plexins have now been implicated in synaptic partner choice in several systems in flies and mammals (Komiyama et al., 2007; Koropouli and Kolodkin, 2014; Matsuoka et al., 2011; Matthes et al., 1995; Pecot et al., 2013; Xie et al., 2017; Sun et al., 2013a). In hippocampus, for example, semaphorins play roles in subcellular specificity. Apical dendrites of pyramidal cells in the hippocampus receive inputs from multiple sources, with inputs of distinct types restricted to discrete lamina-restricted segments of the dendritic arbor (Klausberger and Somogyi, 2008). Semaphorin/plexin interactions regulate two aspects of this subcellular specificity. First, restriction of mossy fibers (arising from dentate gyrus) to the stratum lucidum in CA3 depends on interactions of semaphorin 6A with its receptors, plexins-A2 and A4 (Suto et al., 2007). Second, laminar specificity is sharpened by postnatal pruning the mossy fiber infrapyramidal tract in a process requiring Sema3F. In the spinal cord, semaphorins act in a different way, sharpening synaptic connectivity between sensory axons with motoneurons by suppressing the formation of inappropriate synapses (Pecho-Vrieseling et al., 2009; Fukuhara et al., 2013).

Wnts, Netrins, and Semaphorins Regulate Subcellular Specificity in C. elegans

In *C. elegans*, three conserved signaling pathways conspire to restrict synapses to a specific subcellular domain. DA9 neurons synapse with body wall muscle in restricted segments of their long axons (Figure 9B). Netrin/Unc5 signaling restricts synapses to the dorsal-most region of the axon (Poon et al., 2008). Plexin/sema signaling, mediated by *cis* interactions, sets the anterior boundary of presynaptic differentiation (Mizumoto and Shen, 2013). And Wnt, released from skins cells in the posterior, diffuses anteriorly to define the posterior boundary of presynaptic differentiation (Klassen and Shen, 2007). Thus, the highly restricted distribution of presynaptic sites is defined by three inhibitory pathways.

Ephs and Ephrins Act Locally to Sharpen Topographic Maps

The journey of retinal ganglion cell axons to the optic tectum (superior colliculus), ending with the formation of a retinotopic map, is one of the best-studied models for axon guidance. The paradigm was established by Sperry (1963), whose "chemoaffinity" model posits that connectivity depends on molecular matching. These molecules were sought for decades without success. Finally, Drescher et al. (1995) and Cheng et al. (1995) discovered that gradients of Eph receptor tyrosine kinases, and their membrane associated ligands, ephrins, play crucial roles in map formation. They remain foremost among the molecules that Sperry postulated (Cang and Feldheim, 2013).

Sperry's model is generally viewed as pertaining to the guidance of axons to their targets, so Ephs and ephrins have generally been categorized as axon guidance molecules. However, Sperry's hypothesis also emphasized synaptic specificity, and indeed Ephs and ephrins act primarily at short range, generating repellent signals that lead axons to select topographically matched tectal partners (Pfeiffenberger et al., 2006). Selectivity differs from other cases we have described in two ways. First, the molecular distinctions are not among cell types but rather among cells of a single type that occupy different topographic positions along the anteroposterior or dorsoventral axis.

Second, the distinctions are based on quantitative differences in Eph and ephrin levels rather than their qualitative presence or absence. The importance of graded expression raises the possibility that variations in levels of other recognition molecules also contribute to cell-type-specificity in other parts of the nervous system.

Finally, although we have focused on repulsive interactions promoted by Ephs, ephrins, Wnts, and netrins, these proteins can also regulate synaptic choices by adhesive mechanisms (Goldman et al., 2013; Akin and Zipursky, 2016; Colón-Ramos et al., 2007; Park and Shen, 2012; Hansen et al., 2004; Mao et al., 2018). Differences in signaling determine whether intracellular binding results in adhesion or repulsion.

Specific versus Selective Partner Choices

Neurons are quite promiscuous in their ability to form synapses in culture and following experimental manipulations *in vivo*. The intercellular recognition proteins we have discussed appear to act in large part by curbing this promiscuity. They may do so by enforcing stringent requirements to confer all-or-none "specificity," or by biasing synapse formation to promote "selectivity." In a few cases, genetic analyses have provided evidence for selectivity. For example, loss of function mutations in some fly DIPs lead not only to loss of synapses on the appropriate target but also increased numbers of synapses with other neurons (Xu et al., 2018, 2019; Ashley et al., 2019). These and other results support a model in which molecules encode a hierarchy of preferences. It remains unclear whether selectivity or specificity will emerge as the predominant theme—or whether there is a continuum.

Emerging Themes: Molecular Strategies for Synaptic Specificity

Because selective synapse formation involves near-simultaneous choices among many potential partners, it almost certainly requires a large number of cell-type-specific labels. Results summarized above suggest that this diversity arises in at least three ways: recognition molecules are drawn from expansion of many gene families; each recognition molecule can perform multiple functions; and recognition molecules act in combinations.

Small and Large Families Regulate Wiring in Different Ways

The complexity of neuronal wiring led to the proposal that synaptic specificity would be based on huge families of cell surface recognition molecules with exquisite binding specificity (e.g., "area-code hypothesis") (Hood et al., 1977; Dreyer, 1998). When they were first discovered, *Drosophila* Dscam1 and the vertebrate clustered protocadherins appeared to fit the bill. It became clear, however, that their diversity contributes to synaptic specificity largely by mediating self-/non-self-discrimination and self-avoidance (Zipursky and Sanes, 2010). Over the past decade, evidence has accumulated that partner choice is mediated by a smaller families of cell surface proteins.

These larger and smaller families differ in two ways that underlie their distinct functions. First, Dscam1 and clustered protocadherins exhibit stringent homophilic binding specificity, whereas the second class, typified by the DIPs/Dprs and type II cadherins, support both heterophilic and homophilic interactions and exhibit considerable promiscuity in binding (Figure 5). Second, Dscam1 and clustered Pcdh isoforms are expressed in a probabilistic way that endows individual neurons with distinct molecular identities. In contrast, expression of the smaller families is deterministic and shared by all members of particular neuronal types. Thus, Pcdhs and Dscam1 distinguish individual neurons from each other while the smaller families distinguish neuronal types from each other.

Why might nature have chosen to use multiple families to mediate synaptic specificity rather than massive diversification of fewer families? One possible reason is that selective recognition relies on co-evolution of protein recognition sites and cell-type-specific expression. This may be more readily accomplished through generation of multiple small families than through co-evolving diverse recognition specificities into a single family. Alternatively or in addition, proteins of different families can engage a broader spectrum of signal transduction pathways than could be recruited by large families in which multiple isoforms act through shared intracellular domains.

Multifunctionality

Beyond the large number of recognition molecules, diversity is increased by the ability of many of these proteins to function in different ways in different contexts. Results summarized above reveal at least three types of multifunctionality. First, the same molecule can bind both homophilically and heterophilically, with distinct consequences for partner choice. Second, many recognition molecules can promote both adhesion and repulsion. This feature is so common that one should probably no longer refer to particular recognition molecules as being generally "adhesive" or "repulsive." Third, different levels of a single recognition molecule can convey different messages.

This multifunctionality is less surprising when one realizes that most of the "smaller" gene families we have described likely evolved from genes that regulate development in many cell types. Wnts, Toll, and teneurins, for instance, regulate formation of the embryonic axes, convergent extension, regionalization of the neural tube, axon guidance, and finally cellular and subcellular aspects of synaptic choice (see, for example, Paré et al., 2014, 2019). In this regard, it is intriguing to consider the possibility that some recognition molecules affect partner choice by mechanisms that extend beyond conventional views of specificity. For example, vertebrate Dscams, clustered protocadherins, DIPs, and Dprs all promote neuronal survival (Wang et al., 2002; Lefebvre et al., 2008; Chen et al., 2012). This seemingly distinct function could be related to partner choice in some case. For example, competitive interactions between neurons for DIP/Dpr mediated survival appear to play a critical role in circuit assembly by controlling the number of postsynaptic target neurons of different classes (Xu et al., 2018; Courgeon and Desplan, 2019).

Combinatorial Mechanisms

Combinatorial mechanisms may be the rule rather than the exception in explaining synaptic specificity. At least three types of combinations have been described. First, multiple pathways

may act in parallel to control synapse development, as we saw for Wnt, Semaphorin, and Netrin in *C. elegans*. Alternatively, multiple proteins may form specific complexes that inform connectivity. In the hippocampus, for example, FLRT3 and Ten-2 bind to separate domains in the postsynaptic receptor, Lphn3. Third, multiple members of single families may confer more selective adhesive properties on neurons than can be achieved with single members, as seen for multimers of the clustered protocadherins.

Open Questions

Given the rudimentary state of knowledge in the field, the number of unanswered questions is large. Here, we highlight three that are only beginning to be tackled.

What Is the Relationship between Synaptic Specificity and Synaptogenesis?

We have arbitrarily distinguished synaptic specificity from synaptogenesis, but the extent to which these steps are distinct remains unclear. In one model, initial recognition between synaptic partners precedes and enables the engagement of broadly expressed synaptic organizing molecules (Südhof, 2017, 2018; Kurshan and Shen, 2019; Yuzaki, 2018). Classical cadherins may function in this way (Yamagata et al., 2018; Dagar and Gottmann, 2019). In other cases, however, synaptic organizing molecules selectively expressed by neuronal subsets could function in both specificity and synaptic assembly. Discriminating between these and "hybrid" possibilities *in vivo* may require live imaging *in vivo* as well as development of culture systems that exhibit not only synaptogenesis but also robust specificity.

How Many Molecules Does It Take to Specify a Synaptic Pair?

There are three reasons to suspect that the number is large. First, null mutants in many genes that contribute to specificity have low penetrance or incomplete phenotypes (Xu et al., 2018, 2019). Second, synaptically connected neurons generally express multiple ligand-receptor pairs (Tan et al., 2015). Third, in several cases, genetic analysis has already revealed multiple molecules that contribute to specificity (Duan et al., 2018).

What does this embarrassment of riches imply? One possibility is that important processes are overdetermined—evolution may have implemented multiple paths in case one is disabled. Second, recognition molecules may act in multi-molecular complexes, such that loss of any component reduces, but does not abolish, efficacy. Third, important variations in synaptic structure or function may be determined by different pathways acting in parallel. Inactivation of a single pathway may compromise synaptic structure or function resulting in subtle phenotypes. More incisive ways to characterize synaptic assembly, specificity, and function at high resolution will be necessary to distinguish among these possibilities.

What Is the Contribution of Neural Activity to Synaptic Specificity?

The importance of neural activity for late steps in neural development is clear. For example, absolute and relative activity levels regulate refinement and maintenance of synaptic patterns (Okawa et al., 2014; Valdes-Aleman et al., 2019). It is also clear that elaborate patterns of synaptic connectivity can form in the absence of neural activity (Harrison, 1904; Verhage et al., 2000). However, at least in the mouse olfactory system, different patterns of spontaneous activity result in the expression of different cell adhesion molecules that, in turn, regulate wiring by promoting convergence of axons of the same sensory neuron type to a shared synaptic target (Imai et al., 2006; Serizawa et al., 2006; Nakashima et al., 2019). These and other results raise the possibility that neural activity may affect circuit assembly in qualitative as well as quantitative ways.

Concluding Comment

Despite considerable progress in understanding early steps in neural development, we are a long way from understanding how the neural circuits are assembled that underlie perception, cognition, emotion, action, and much more. However, new tools provide enormous opportunities to solve the wiring problem. We anticipate that critical insights and principles will emerge over the next few decades through creative combinations of genetics, biochemistry, structural biology, imaging, and physiology. As we reflect on the accelerating progress of the last decade, we are reminded of Churchill's words: "This is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning."

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