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Visual circuit development in *Drosophila*

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Fly visual circuits are organized into lattice-like arrays and layers. Recent genetic studies have provided insights into how these reiterated structures are assembled through stepwise processes and how precise connections are established during development. Afferent-derived morphogens, such as Hedgehog, play a key role in organizing the overall structure by inducing and recruiting target neurons and glia. In turn, the target-derived ligand DWnt4 guides Frizzled2-expressing photoreceptor afferents to their proper destination. Photoreceptor afferents select specific synaptic targets by forming adhesive interactions and regulating actin cytoskeleton in growth cones. Target specificity is probably achieved by restricting the expression of adhesive molecules, such as Capricious, to appropriate presynaptic and postsynaptic partners, and by differentially regulating the function of broadly expressed adhesive molecules such as N-cadherin.

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Introduction

The visual circuits of the fly are capable of sophisticated functions such as motion detection, pattern recognition and color vision [1–3]. Reflecting their common functional requirements, fly and vertebrate visual circuits share several features of organization, including a retinotopic map and layer-specific connectivity. This similarity supports the notion that studying the development of fly visual circuits could shed light on the more complex vertebrate counterpart. Moreover, the fly visual system provides several attractive features.

First, the circuits are genetically hardwired. The connections are well characterized anatomically and are essentially invariant from fly to fly. Second, the molecular and cellular processes of eye development in flies have been

studied in great detail and many useful tools have been developed. These tools, in combination with generic fly genetic methodology, make possible high-resolution phenotypic analysis and sophisticated genetic manipulation. Last, behavioral assays are available both for genetic screens and for correlating structural defects with functional deficits (reviewed in [4]).

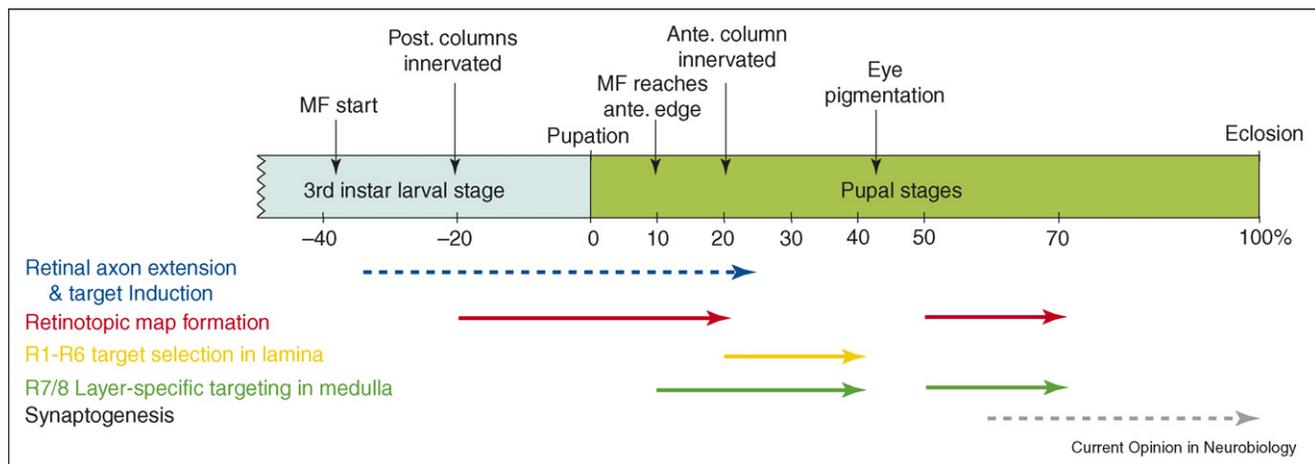
In recent years, significant advances have been made in understanding the development of the fly visual circuits at the cellular and molecular level. In particular, forward genetic screens have identified several key molecules that establish retina–brain connectivity, and the mechanisms of their action are now being elucidated (reviewed in [5]). Here, we focus on the development of connections made by photoreceptor afferents. We first describe briefly the anatomy of the visual circuits, and then discuss the development of specific organizational features (Figure 1) and their molecular and cellular mechanisms.

The anatomy of the fly visual system

The fly visual system comprises the retina (compound eye) and four optic ganglia known as the lamina, the medulla, the lobula and the lobula plate (Figure 2a) [6]. The retina comprises approximately 750 unit eyes, termed ‘ommatidia’, which are arranged in a hexagonal crystalline array. Each ‘ommatidium’ has eight photoreceptor neurons (R cells), which are further divided into three subtypes determined by morphology, position in the ommatidium and *opsin* gene expression. R1–R6, the outer photoreceptor neurons, express the opsin Rh1, which responds to a broad spectrum of light with a maximum in the green color range. R1–R6 are thought to be functionally equivalent to vertebrate rod cells and are required for detecting motion. The inner photoreceptors R7 and R8 have more complex patterns of *opsin* gene expression: R7 photoreceptors express the ultraviolet-sensitive Rh3 or Rh4 opsin, whereas R8 photoreceptors express the blue-sensitive Rh5 or green-sensitive Rh6 opsin (Figure 2b) [7]. R7 and R8 photoreceptors are required for color vision and represent the closest analog of vertebrate cone cells.

Photoreceptor axons from a single ommatidium form a bundle to innervate the lamina and medulla in a retinotopic fashion. The R1–R6 axons innervate the lamina, whereas the R7 and R8 axons project through the lamina and terminate at two different layers in the medulla ganglion (Figure 2b) [6]. In the lamina, R1–R6 axons project in a stereotyped pattern to connect to lamina neurons to form a synaptic unit, called a ‘cartridge’ [6]. The projection pattern of the R1–R6 axons reflects the

Figure 1



Developmental timetable of the fly visual system. Various organizational features in the visual system develop concurrently (arrows). The exact development time for target induction and synaptogenesis is not known (broken arrows). The pupal stage spans ~100 h at 25 °C; thus, 1% is roughly equivalent to 1 h in developmental time. Several key events in the development of the eye and optic lobe are indicated. Abbreviation: MF, morphogenetic furrow in the eye disc.

organization of their rhabdomeres (light-sensing structures) in the ommatidium, and its purpose is to pool the visual input from the R1–R6 neurons from six separate ommatidia (Figure 2b).

All visual information converges on the medulla ganglion, which is organized into layers and columns. Each column receives input directly from R7 and R8, and indirectly from R1–R6 through lamina neurons, all of which receive visual information from a single point in visual space [6]. Each column is further divided into ten layers, termed M1–M10. The R8 and R7 axons terminate at, respectively, the M3 and M6 layers, whereas lamina neurons connect to various M1–M5 layers (Figures 2b and 3). The layer-specific connections made by the R7, R8 and lamina neurons are reminiscent of those observed in the vertebrate cortex. Staining of the Golgi has revealed about 50 subtypes of medulla neurons that interconnect different medulla layers or connect the medulla to higher visual ganglia — namely, the lobula and lobula plate [8]. Each subtype of medulla neuron extends dendritic arbors and axons to specific layers of the medulla (or lobula or lobula plate) in stereotyped patterns; however, the connectivity of the medulla is not known in detail. The higher optic ganglia — the lobula and lobula plate — are organized in columns and layers and form interconnections with the medulla in a topographic manner. Because very little is known about their development, they are not discussed further here.

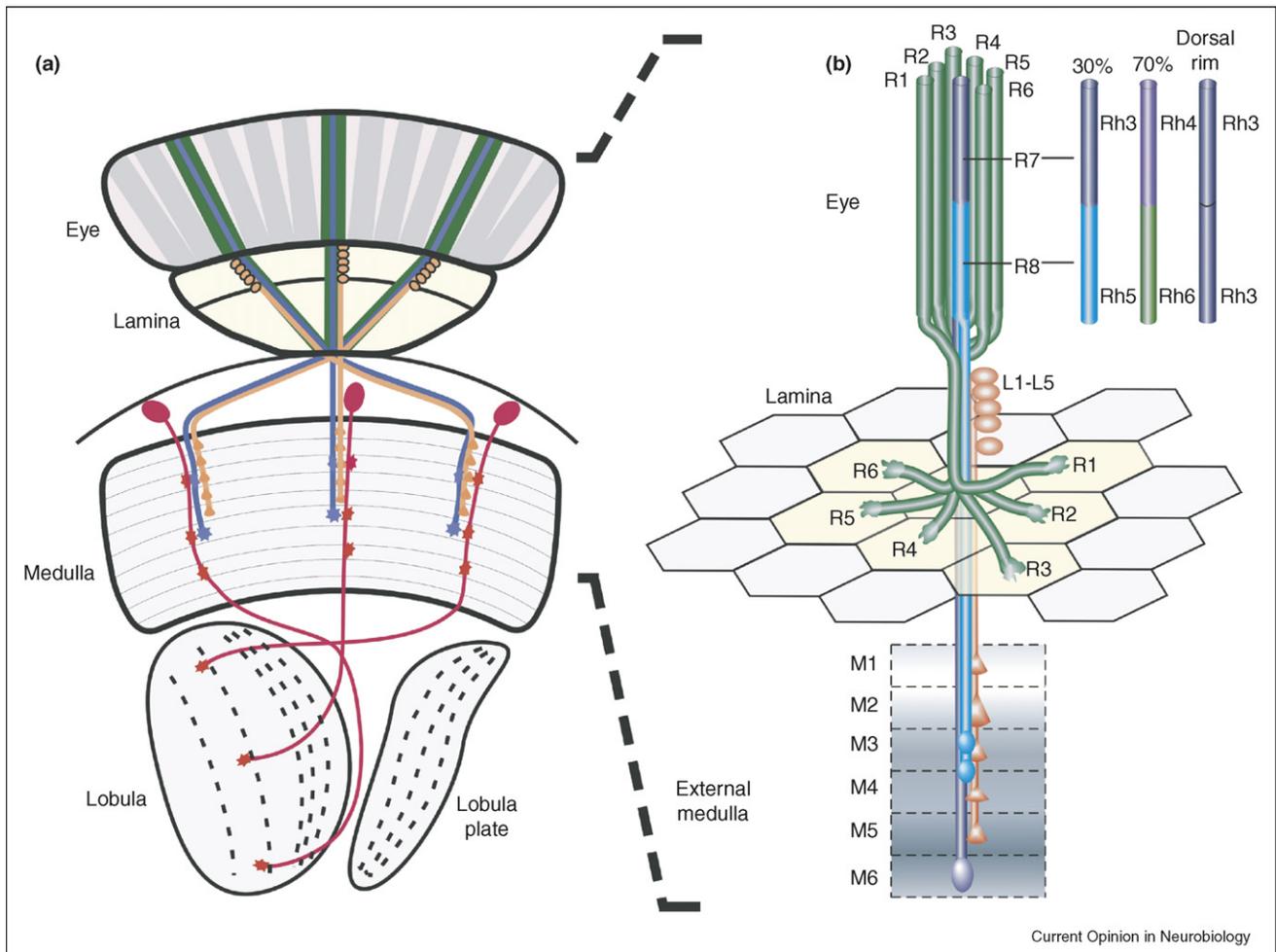
Early development: Inducing target development and recruiting neurons and glia

Although the importance of retinal innervation for optic lobe development has long been recognized, the mechanisms by which retinal afferents coordinate the

development of their target are only now being elucidated. As the morphogenetic furrow sweeps from the posterior to the anterior edge of the developing eye discs, rows of photoreceptor neurons sequentially differentiate and project axons into the optic lobe. The arriving photoreceptor axons orchestrate the development of the optic lobe. First, each new row of photoreceptor axons delivers Hedgehog and an epidermal growth factor (EGF)-like ligand, Spitz, to induce the proliferation and differentiation of lamina neurons, which then associate with the photoreceptor axons to form lamina columns (or cartridges) [9,10]. Consequential to these repeated inductive events, the number of lamina neurons matches the number of arriving photoreceptor afferents; thus, the photoreceptor axons are separated by rows of lamina neurons to form a regular array (see below for formation of the retinotopic map) [11]. Second, the photoreceptor afferents, through an unidentified mechanism, induce the outgrowth of scaffold axons, which in turn act as a substrate for glia migration [12].

By means of four distinct sets of scaffold axons, different subtypes of glia migrate to specific regions in the optic lobe and provide various functions at their destinations [12]. The lamina neuropil glia (including the epithelial and marginal glia) function as intermediate targets for the R1–R6 axons, which follow the R8 axons (see below). In addition, the lamina glia express the repulsive cue Slit to prevent Robo-expressing lobula axons from inappropriately entering the lamina [13]. The medulla neuropil glia are required for the survival of medulla cortex neurons and presumably provide an unknown tropic factor [12]. The molecular cues that guide glia to specific locations, however, remain unknown.

Figure 2



The anatomy of the fly visual system. **(a)** Gross anatomy. The visual system comprises the retina (compound eye) and four ganglia termed the lamina, the medulla, the lobula and the lobula plate. The medulla, lobula and lobula plate are organized into layers. Photoreceptor axons project into the lamina and medulla (green and purple lines) to form synapses with lamina (orange) and medulla (red) neurons, respectively, whereas lamina neurons make connections to the medulla, and medulla neurons connect to the lobula and lobula plate (only 1 of ~50 types of medulla neurons and none of the centrifugal neurons are shown). **(b)** Connection patterns of the photoreceptor neurons. The eye comprises ~750 ommatidia (green columns), each of which contains eight photoreceptor neurons termed R1–R8. The axons of R1–R8 form a common bundle to innervate the lamina and medulla in an invariant pattern. The R1–R6 axons defasciculate from the common bundle at the lamina and extend laterally to form synapses with the lamina neurons in the neighboring columns. The connection pattern of R1–R6 in the lamina is stereotyped and reflects the organization of their rhabdomeres (light-sensing structures) in the eye. The R7 and R8 axons extend through the lamina and terminate in two medulla layers, termed M3 and M6, respectively. Similarly, the lamina neurons (L1–L5) make layer-specific connections in the medulla (see Figure 3c). R1–R6 express a broad-spectrum opsin (Rh1), R7 expresses an ultraviolet-sensitive opsin (Rh3 or Rh4), and R8 expresses a blue-sensitive (Rh5) or green-sensitive (Rh6) opsin. In most parts of the retina, the expression of opsin by R7 and R8 is coordinated: that is, in a single ommatidium, Rh3-expressing R7 is coupled with Rh5-expressing R8; and Rh4-expressing R7 is coupled with Rh6-expressing R8. In the dorsal rim ommatidia, which specialize in polarized light detection, both R7 and R8 express Rh3.

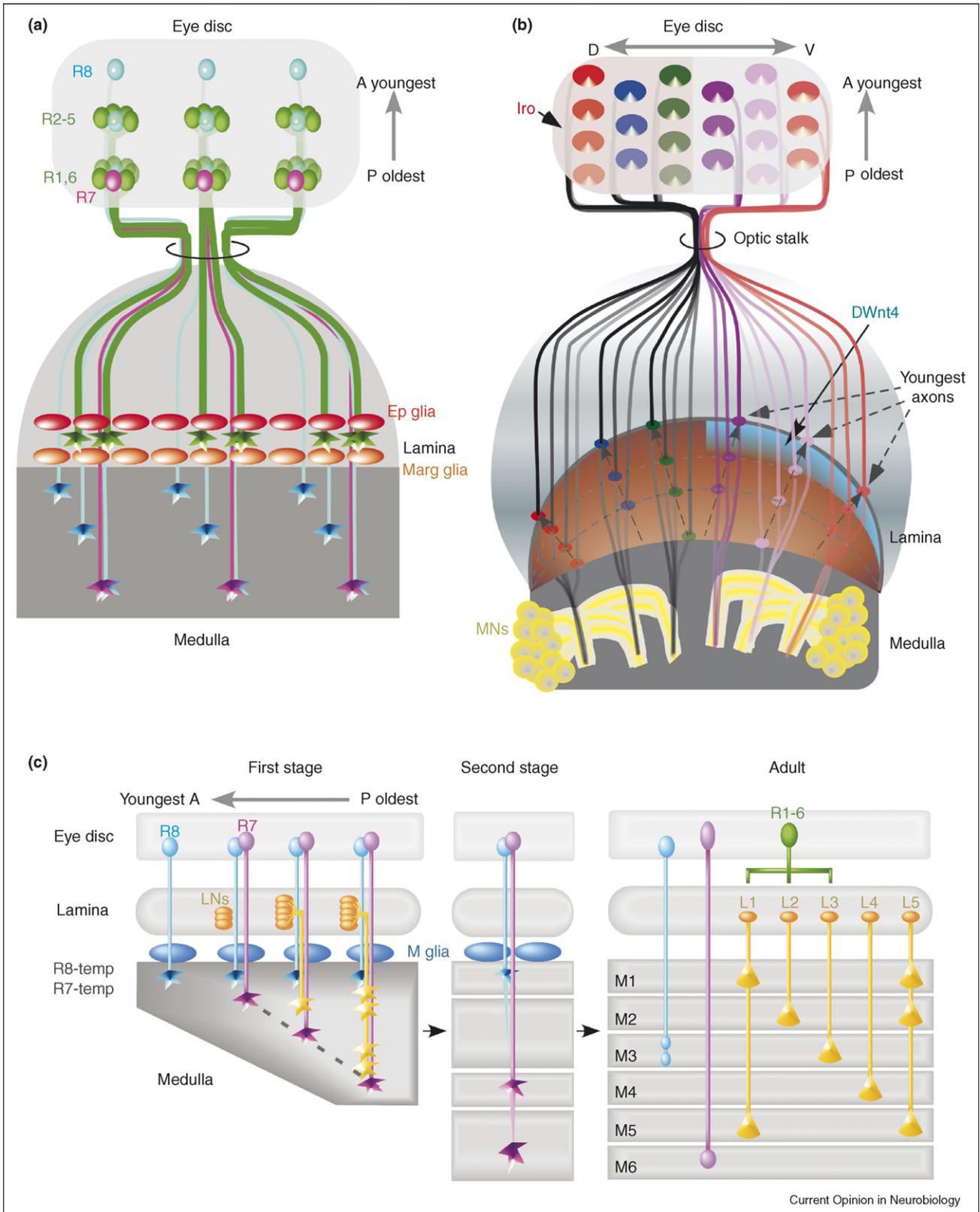
Ganglion specificity: guiding photoreceptor afferents to the neuropil

Within each photoreceptor cluster, R8 differentiates first and projects axons into the medulla. The R1–R6 axons follow the pioneering R8 axons into the brain but stop at the lamina ganglion, whereas the R7 axons project past the lamina and terminate at the medulla ganglion (Figure 3a). The correct targeting of R1–R6 and R7–R8 to different ganglia reflects specific properties of their growth cones

rather than the timing of their axon outgrowth. Lamina glia function as intermediate targets for R1–R6 axons, presumably by providing a ‘stop’ signal. Indeed, disruption of glial migration, as observed in mutants of *non-stop* or *JAB1* (also known as *CSN5*), results in most R1–R6 axons extending beyond the lamina into the medulla neuropil [14,15].

Several genes have been identified for proper axonal termination of R1–R6 at the lamina. Mutations in the

Figure 3



Development of the organizational features of the visual system. **(a)** Ganglion specificity. Different subtypes of photoreceptor extend axons to different optic ganglia at the third instar larval stage. R8 photoreceptors are the first to differentiate, and they project axons (blue) that terminate at the medulla

transcription factor Brakeless (also known as Scribbler) cause misregulation of the transcription factor Runt in R2 and R5, leading to many R1–R6 axons failing to stop at the lamina [16–18]. These transcription factors probably function indirectly by regulating the expression of unidentified receptors or signaling components that are required for response to the glial signal.

Previous studies have identified several signaling molecules that might be downstream targets of the above-mentioned transcription factors. These molecules include the adaptor protein Dreadlocks (also known as Dock), the receptor tyrosine phosphatase PTP69D, the receptor tyrosine kinase Off-track, and the serine/threonine kinase Misshapen, all of which are required in R1–R6 for lamina ganglion specificity [19–24]. Their mutant phenotypes are less severe than those of the glia migration mutants, however, which presumably reflects a high degree of redundancy among the signaling mechanisms. Some of these mutations also disrupt glia configuration and could potentially disrupt discharge of the stop signal, thereby indirectly affecting lamina ganglion specificity. At present, the precise functions of the identified signaling molecules remain unclear, and the molecular nature of this stop signal is not known. A directed genetic mosaic system that facilitates genetic manipulation of glial cells has been recently developed and could facilitate investigation of this glia-derived signal [25*].

Retinotopic map formation: preserving the spatial relationship of photoreceptors

The retinotopic map — the hallmark of visual system connectivity — is crucial for preserving spatial information during processing. Thus, it is not surprising that several mechanisms are used to ensure the formation of a precise map along both the anterior–posterior and dorsal–ventral axes (Figure 3b). The formation of an initial map of photoreceptor termini is coupled with eye differentiation and successive waves of axonal projection into the brain along the anterior–posterior axis. The sequential induction and recruitment of lamina neurons ensures that there is proper spacing between axon bundles [10,11].

Dorsal and ventral photoreceptor axons are known to have distinct properties that enable them to reach their corresponding brain regions [26]. A recent study on signaling by DWnt4 and Frizzled2 has now revealed the molecular mechanism involved in this process. In the ventral half of the eye disc, photoreceptor afferents express Frizzled2 and respond to DWnt4 expressed in the ventral target region; by contrast, Frizzled2 function in the dorsal half of the photoreceptors is presumably suppressed by the homeobox transcription factor Iroquois to prevent these axons from entering the ventral target region [27**]. In this context, Frizzled2 functions through the non-canonical Wnt pathway through the adaptor protein Disheveled. Unlike vertebrate Eph and Ephrin, which are expressed in gradients in retinal afferents and their target region (the tectum), respectively, DWnt4 and Iroquois have binary expression patterns along the dorsal–ventral axis. Thus, they probably provide only rough positional information.

The formation of a precise retinotopic map of R8 termini involves adhesion-mediated fasciculation and cytoskeletal regulation in the growth cones. The homophilic protocadherin Flamingo probably matches the R8 axons to medulla axons as they project into the medulla [28]. Most importantly, the insulin signaling pathway has been shown to maintain proper spacing between axons: the insulin receptor functions through the adaptor protein Dock and through cytoskeletal regulators including Pak, Trio and LIM kinase [19,29–31]. The identification of these cytoskeletal regulators as downstream effectors of the insulin signaling pathway underscores the importance of proper regulation of the actin cytoskeleton in retinotopic map formation. The identities of the insulin receptor ligand and its cell source remain unknown and their discovery will be crucial for fully understanding the function of this important pathway. Furthermore, Semaphorin-1a and acetylcholine seem to be required for proper spacing of photoreceptor axons, but their functions remain poorly defined [32,33].

During the early development stages, R7 axons follow the pioneering R8 axons to the medulla. Thus, the initial R7

(Figure 3 Legend Continued) (grey box). The R1–R6 axons (green) follow the R8 axons but stop at the lamina. R7 photoreceptors, the last to differentiate, project axons into the medulla. Thus, the ganglion choice made by photoreceptor subtypes does not simply follow their order of innervation but reflects their growth cone properties. Lamina glia cells serve as intermediate targets for R1–R6 axons by providing a so far unidentified ‘stop’ signal. **(b)** Retinotopic map formation at the third instar larval stage. Several cellular mechanisms are used to establish the retinotopic arrangement of photoreceptor axons along the anterior–posterior (A–P) and the dorsal–ventral (D–V) axes. First, successive waves of photoreceptor afferents sequentially induce and recruit lamina neurons (not shown). Second, the newly arriving photoreceptor axons (darkest lines) fasciculate with medulla axons (yellow) by expressing matching adhesive molecules such as Flamingo. Third, the ventral photoreceptor afferents are guided by DWnt4, which is expressed in the ventral half of the lamina (blue stripe); by contrast, the dorsal retina expresses Iroquois (Iro), which presumably renders DWnt4 signaling ineffective. The anterior-most photoreceptors are the youngest (darkest discs), and they project axons into the outermost edge of the lamina (brown crescent). **(c)** Layer-specific targeting. The R8, R7 and lamina axons target specific layers in two distinct stages. Left, during the first target-selection stage, the R8 (blue), R7 (purple) and L1–L5 (yellow) axons sequentially project into distinct layers in the medulla. R8 differentiates first and extend axons into the superficial medulla layer (R8-temporary layer). The R7 axons follow the R8 axons and terminate at a deeper layer (R7-temporary layer) just below the R8 growth cones. Lamina neurons are the last to differentiate, and their growth cones terminate between the R7 and R8 growth cones to separate them. The arrow indicates the developmental sequence in the eye disc. In this view, rotated 90° from that in (b), the younger R7 and R8 growth cones are to the left and the older ones are to the right. Middle, at the second target-selection stage, both the R7 and R8 growth cones regain motility to proceed to their destined layers. For clarity, the R1–R6 and L1–L5 neurons have been omitted. Right, at the adult stage, the R7, R8 and L1–L5 axons innervate specific medulla layers, as indicated. Abbreviation: M. glia, medulla glia (blue).

retinotopic map simply reflects the R8 map. However, the R7 retinotopic map is refined at the late pupal stage and this refinement requires competitive interactions among R7 termini [34], as well as intrinsic control of growth cone motility (C-Y Ting and C-H Lee, unpublished data). A recent study suggests that Activin signaling might be involved in this process (C-Y Ting and C-H Lee, manuscript in preparation).

Synaptic target selection: R1–R6 selection of lamina cartridges

After reaching the lamina neuropil, the R1–R6 axons segregate from common bundles and extend laterally in a stereotyped pattern to innervate their neighboring cartridges (Figure 2b, green axons) [6]. The establishment of R1–R6 connections does not require synaptic vesicle release or electrical activity, suggesting that these connections are genetically hardwired [35[•]]. Ultrastructural studies have demonstrated that the R1–R6 growth cones form distinct stereotyped interactions with one another at different stages [6]. In addition, elegant genetic manipulation of ommatidial orientation and R1–R6 subsets has shown that interactions among the R1–R6 axons within the ommatidial bundle are essential for R1–R6 target selection [36]. Although these studies suggest that afferent–afferent interactions have a key role in directing the R1–R6 axons to appropriate cartridges, the molecules that mediate these interactions remain elusive.

The importance of afferent–target interaction in directing R1–R6 target selection has been underscored by the identification of four molecules: namely, two adhesion molecules, N-cadherin and the protocadherin Flamingo; the receptor tyrosine phosphatase LAR; and the scaffolding protein Liprin- α . N-cadherin, LAR and Liprin- α are required at an early developmental stage for radical segregation of the R1–R6 growth cones from common bundles and for their lateral projection to the cartridge [37–39]. Mosaic analysis has shown that N-cadherin is required in both R1–R6 and lamina neurons, suggesting that N-cadherin mediates homophilic interactions between afferents and targets [40[•]]. Because N-cadherin is expressed in all photoreceptor axons, its activity might be asymmetrically regulated so that the interaction between photoreceptor axons in the ommatidial bundles is weakened, thereby enabling each R1–R6 axon to project laterally to the appropriate cartridge [39]. LAR and Liprin- α share similar phenotypes with N-cadherin. Unlike N-cadherin, however, they are required only in R1–R6 and might regulate N-cadherin activity at the presynaptic sites. By contrast, Flamingo mutants show very different R1–R6 phenotypes from those of N-cadherin, LAR or Liprin- α mutants, suggesting that this protein functions by a distinct mechanism [28]. The mechanism of its action, however, remains unclear.

Once they have reached the appropriate cartridge, the R1–R6 axons form synapses with lamina neurons (L1–L3) [41]. The number of synapses that each R1–R6 terminal makes is constant in all cartridges, including those in the margin or equator where, respectively, naturally fewer or more than six R1–R6 termini are received [42]. This remarkable constancy has been demonstrated by the analysis of a large collection of known and novel mutants that affect R1–R6 sorting [35[•]]. The fact that R1–R6 makes a constant number of synapses, regardless of the cartridge composition, strongly suggests that the synapse frequency is determined presynaptically.

Layer-specific target selection: axon targeting to the medulla layers

The R8, R7 and lamina neuron (L1–L5) axons terminate at specific layers of the medulla (Figure 3c). Developmental analysis has shown that these axons project to specific layers in two distinct stages: during the first (early pupal) stage, the R8, R7 and lamina axons sequentially project to their temporary layers; during the second (late pupal) stage, their growth cones regain motility and progress synchronously to their destined layers [43^{••}]. Genetic ablation of R7 or lamina neurons, coupled with alteration of R8 connectivity, has revealed that these afferents target their temporary layers independently. Thus, afferent–target, but not afferent–afferent, interactions dictate the targeting specificity at the first target-selection stage [43^{••}]. At the early pupal stage, some medulla neurons have extended dendritic arbors in the R7- or R8-temporary layers, suggesting that they might act as temporary targets. The identity of these neurons is still uncertain. In addition, the synchronous extension of R7 and R8 growth cones at the second target-selection stage suggests that it is probably triggered by a global signal, the identity of which remains unknown.

Genetic screens based on visual behavior or histology have identified several surface molecules involved in layer-specific targeting by R7, including the adhesion molecule N-cadherin, the receptor tyrosine phosphatases PTP69D and LAR, and the adaptor protein Liprin- α . Mutations in N-cadherin, LAR or PTP69D cause the R7 axon to mis-target to the R8-recipient layer [22,37,38,44,45[•]]. N-cadherin mediates homophilic interactions between R7 growth cones and medulla target neurons (S Yonekura, submitted). Developmental analysis has revealed that N-cadherin is required for R7 growth cones to reach and to remain in the R7-temporary layer at the first target-selection stage and subsequently to extend to the destined layer at the second stage [43^{••},46]. By contrast, LAR and Liprin- α function only at the later target-selection stage. LAR and Liprin- α physically interact, and genetic epistasis experiments place Liprin- α downstream of LAR. Liprin- α and LAR probably work together to modulate the actin cytoskeleton through the Rho GTPases regulators Enable and Trio [44,45[•]]. The function of PTP69D in R7 target

selection is not known. N-cadherin, LAR, PTP69D and Liprin- α are broadly expressed, suggesting that they must be differentially regulated in the R7 growth cones in order to exert specific functions.

Capricious, a leucine-rich adhesion receptor, has an instructive role in layer-specific targeting by R8. Both gain-of-function and loss-of-function studies indicate that Capricious directs R8 axons to the appropriate medulla layer [47^{**}]. Capricious can mediate homophilic interactions and has an informative expression pattern: it is expressed in R8 (but not R7) photoreceptors and in a subset of medulla neurons that extend processes in the R8-recipient layer. Thus, Capricious probably mediates afferent–target interaction. In addition, Flamingo and N-cadherin have been shown to be involved in R8 layer-specific targeting [43^{**},48]. Both molecules are broadly expressed and have pleiotropic functions in other photoreceptors. Whereas N-cadherin might provide permissive interaction between R8 and medulla neurons, the function of Flamingo in this context remains unclear.

Conclusion

Significant progress has been made in our understanding of the development of connections made by photoreceptor neurons. Many guidance molecules have been cloned and characterized, and some of their signaling pathways have been delineated. These advances, however, raise even more challenging questions with broad relevance. How do photoreceptor growth cones integrate multiple guidance signals? How do photoreceptor afferents select specific synaptic partners and form synapses? How are highly diverse medulla neurons generated? How do different medulla neurons establish stereotyped dendritic arbors? How do visual circuits evolve to adapt new functions?

Studies of visual circuit development have uncovered many novel cellular and molecular mechanisms in the past, and will no doubt continue to provide insights into the development of the central nervous system in general in the years to come.

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