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Missed connections: photoreceptor axon seeks target neuron for synaptogenesis

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Extending axons must choose the appropriate synaptic target cells in order to assemble functional neural circuitry. The axons of the *Drosophila* color-sensitive photoreceptors R7 and R8 project as a single fascicle from each ommatidium, but their terminals are segregated into distinct layers within their target region. Recent studies have begun to reveal the molecular mechanisms that establish this projection pattern. Both homophilic adhesion molecules and specific ligand–receptor interactions make important contributions to stabilizing R7 and R8 terminals in the appropriate target layers. These cell recognition molecules are regulated by the same transcription factors that control R7 and R8 cell fates. Autocrine and repulsive signaling mechanisms prevent photoreceptor terminals from encroaching on their neighbors, preserving the spatial resolution of visual information.

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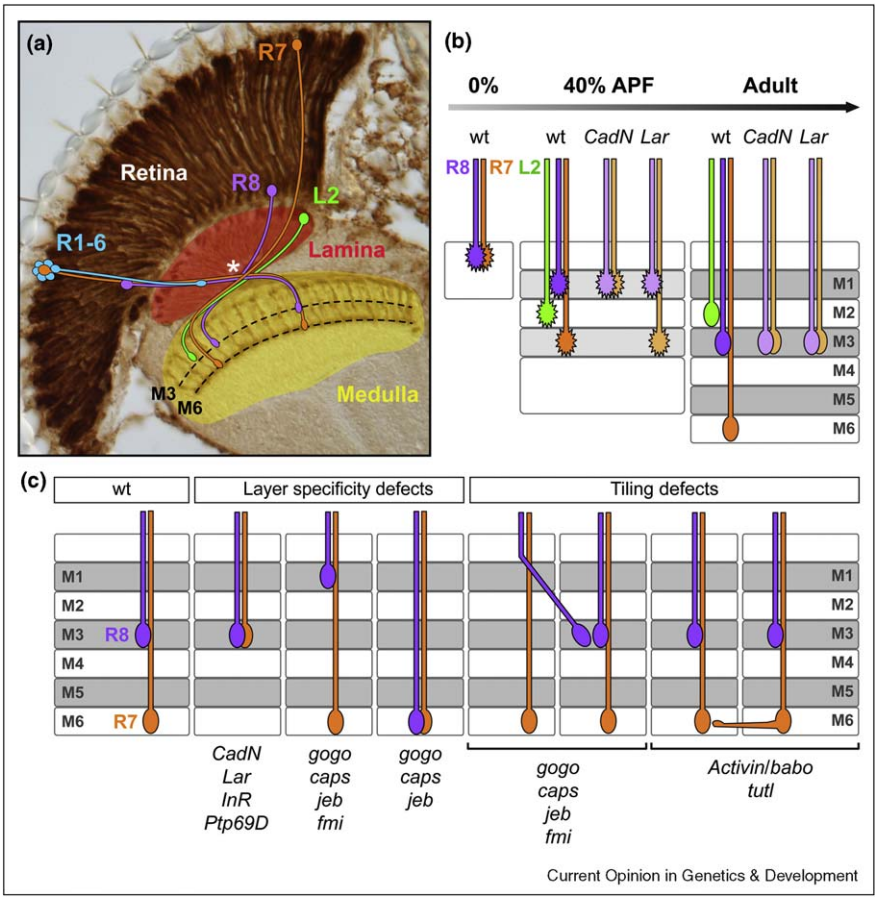
Introduction

Formation of a functional nervous system requires developing neurons to extend processes to the appropriate target regions and then select the correct synaptic partners. Synapses between particular cell types are frequently localized to a specific layer within a complex neuropil, even when the cells extend processes through or into other layers. In addition, preservation of spatial information is often achieved by non-overlapping columnar organization of the projections from a field of cells. While such layer-specific and columnar connections are a common feature of the mammalian cerebral cortex [1,2], their molecular basis is easier to investigate in simpler model systems. One such system that has been particularly fruitful is target selection by the two photoreceptors that mediate color vision in *Drosophila*.

Each of the 800 ommatidia of the *Drosophila* compound eye contains 8 photoreceptor cells, which project their axons directly into the brain in a retinotopic manner. The outer photoreceptors R1–R6 all express the same Rhodopsin and project into the first optic neuropil, the lamina, which mediates image formation and motion detection [3,4] (Figure 1a). By contrast, the inner photoreceptors R7 and R8 each express one of four Rhodopsins with different spectral sensitivities that form the basis for color perception; R7 cells sense ultraviolet (UV) light and R8 cells blue or green light [5]. The R7 and R8 photoreceptors from one ommatidium project their axons as a single column into the second optic neuropil, the medulla, where their terminals are segregated into two distinct layers: R8 terminates in the M3 layer, while R7 projects to the deeper M6 layer [3] (Figure 1a). R8 differentiates and extends its axon first, potentially forming a substrate for the R7 growth cone to follow; nevertheless, the R7 axon terminates in the correct target layer even when the R8 axon fails to enter the medulla [6•,7••]. Targeting occurs in two steps; the growth cones of both R7 and R8 pause in more superficial, but again separate, temporary layers before reaching their final target layers in late pupal stages [8•] (Figure 1b).

This process of target layer selection might be explained by Sperry's chemoaffinity hypothesis [9], which states that neurons recognize their target cells using specific molecular labels. However, layer-specific distribution of such labels in the medulla is complicated by the presence of many different classes of interneurons and projection neurons [10], some of which arborize in both the R7 and R8 target layers [11,12,13•]. Some medulla neurons receive input from the R7 and/or R8 cells of only one ommatidium, while others extend their dendrites over a larger area to receive input from either or both photoreceptor types belonging to multiple ommatidia. Medulla neurons can also integrate information from R1–R6 via synaptic input from lamina neurons, the primary targets of the outer photoreceptors [11,12,13•] (Figure 1a). Molecules important for target layer selection by R7 and R8 might be expressed by classes of cells that arborize in only one layer, perhaps including lamina neurons, which themselves terminate in specific medulla layers [10,14]. Alternatively, they could be broadly expressed, but localized to only those dendrites that extend into the target layer.

Figure 1



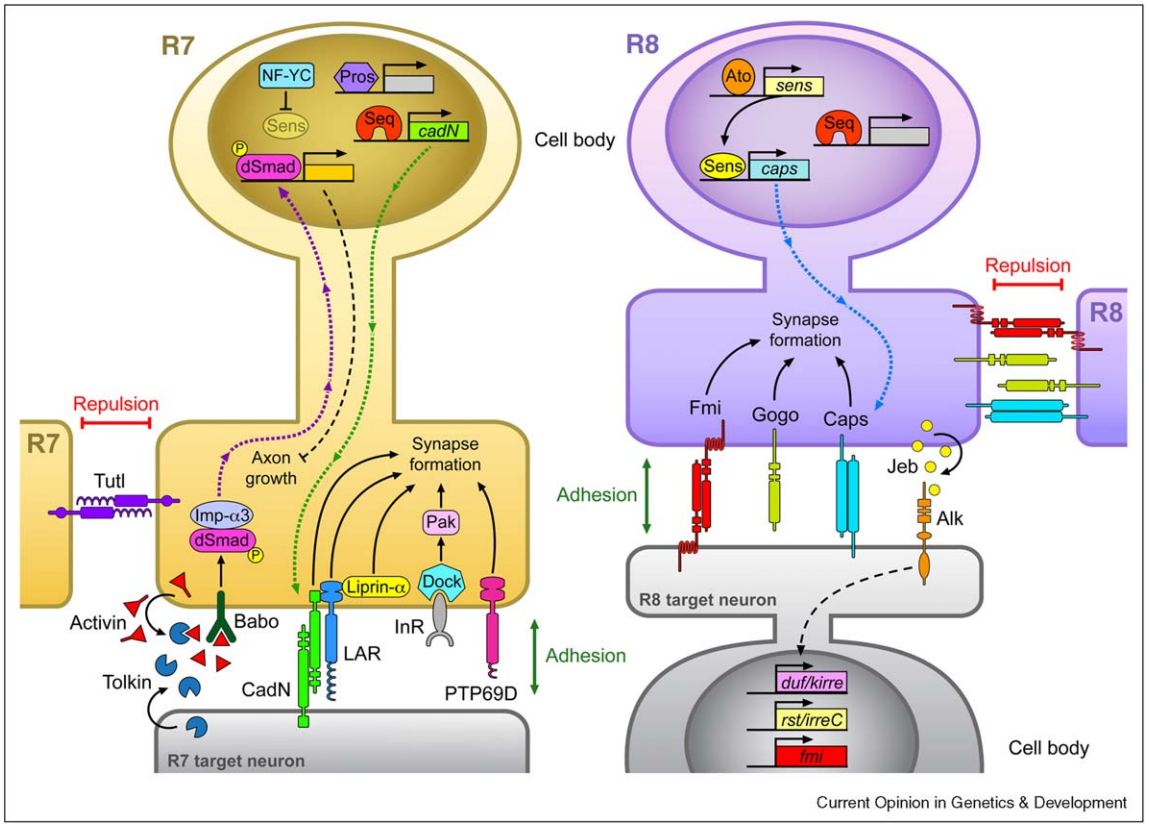
Wild-type and mutant R7 and R8 projection patterns. **(a)** shows *Drosophila* photoreceptor axons labeled with the photoreceptor-specific *glass-lacZ* marker, which is visualized by anti-β-galactosidase staining of an adult head section. Photoreceptor cell bodies are located within the retina; R1–R6 (blue) send their axons into the first optic neuropil, the lamina, while R7 (orange) and R8 (purple) axons terminate in two distinct layers in the second optic neuropil, the medulla. This projection pattern is retinotopic, i.e. photoreceptors from neighboring ommatidia project to neighboring targets in the optic lobes; crossing of fibers in the outer optic chiasm (asterisk) reverses their anterior-posterior orientation in the medulla relative to the lamina. The five classes of lamina neurons also form a retinotopic projection into the medulla, where they terminate in several layers; only L2 (green) is shown here. **(b)** diagrams the establishment of the R7 and R8 projection pattern during development. At pupariation, the growth cones of R7 and R8 have not yet separated. By 40% APF (after puparium formation) R7 and R8 have reached their temporary layers, and ingrowing lamina neuron processes have further separated these layers. CadN, but not LAR, is required for R7 to reach its temporary layer at this stage. The adult projection pattern is established in a second phase that may involve axon growth, passive displacement by ingrowing processes, or both, and that requires both CadN and LAR in R7. The distinct temporary and final target layers were identified by the change in position of the R8 termini with respect to the growth cones of L2 lamina neurons. **(c)** shows schematics of the layer specificity and tiling defects caused by the mutations listed under each column. Only mutations affecting secreted or transmembrane proteins are shown. Some mutations cause multiple phenotypes.

The columnar and layered organization of the R7 and R8 projection pattern is easily visualized by light microscopy, making it accessible to genetic analysis. Both morphological screens and behavioral screens based on the preference of adult flies for UV light, which is sensed by R7 and a population of its target neurons that arborize in the M6 layer of the medulla, the Dm8 interneurons [13•], have been used to identify molecules important for target recognition by the R7 and R8 photoreceptors [15–18,19•]. Use of the FLP-FRT system to generate genetic mosaics in which only photoreceptors [16], only R7 [20], or only lamina and medulla neurons [21] are homozygous mutant has further enabled the site of action of each gene to be determined. This review will discuss our current understanding of target layer selection derived from such genetic screens.

Transcription factors control both cell fate and target layer selection

Photoreceptor identity is determined by combinations of transcription factors that control the expression of both *rhodopsin* genes and molecules necessary for target layer selection. Two transcription factors, Spalt and Runt, are common to both R7 and R8, distinguishing them from the outer photoreceptors [22,23]; however, neither is essential to establish their axon projection patterns. R7 and R8 cells

Figure 2



Molecular mechanisms mediating R7 and R8 axon targeting. In R7 and R8, specific transcription factors regulate the expression of cell surface molecules important for recognition of the correct target cells. In R7 cells, Pros and NF-YC activate the expression of R7 genes and repress the expression of R8 genes respectively, while Ato activates the expression of Sens in R8. *seq* expression first in R8 and then in R7 positively regulates the expression of genes that include *cadN*. In R7, the cell surface proteins CadN, LAR, PTP69D, InR and Babo contribute to synapse formation and stability through homophilic or heterophilic interactions with the target neurons. The transmembrane proteins Caps, Fmi, and Gogo, and the secreted protein Jeb and its receptor Alk, contribute to the termination of R8 axons in the correct recipient layer. Homophilic interactions of Tutl in R7, and Caps and Fmi in R8, seem to mediate repulsion between the axons in neighboring columns that maintains the normal spacing of R7 and R8 projections.

lacking the two *spalt* genes resemble R1–R6 in forming large rhabdomeres and expressing Rh1, but form normal projections into the medulla [22]. Although loss of *runt* has no phenotype, possibly owing to redundancy with related genes, Runt misexpression can transform outer photoreceptors into cells with the rhabdomere morphology, *rhodopsin* expression, and axon projection pattern of R7 and R8 cells [23,24]. Such misexpression occurs naturally in the absence of the transcriptional repressor Brakeless [23,25].

The distinction between the R7 and R8 fates is primarily controlled by two cell type-specific transcription factors, Senseless (Sens) in R8 and Prospero (Pros) in R7 [26–28]. Sens is expressed specifically in R8 owing to activation by the proneural transcription factor Atonal and repression in other photoreceptors by Rough [26], while Pros expression in R7 depends on a combination of eye-specific transcription factors and signals received from neighboring cells [29]. Both the presence of Pros and the absence of Sens in R7 are important for its normal targeting (Figure 2). Most *pros* mutant R7 cells form expanded termini in both the M3 and M6 layers, suggesting that these cells take on an intermediate identity consistent with their lack of Sens expression [30]. By contrast, R7 cells that lack the C subunit of the transcription factor nuclear factor Y (NF-YC) do ectopically express Sens, which causes them to mistarget to the M3 layer [31••]. One target of Sens in this process is the R8-specific adhesion molecule Capricious (Caps) [32••], but additional target genes must be involved, since removal of *sens* from *NF-YC* mutant R7 cells rescues their mistargeting, but removal of *caps* does not [31••]. Late loss of *sens* in R8 causes a variety of targeting defects [31••], indicating that Sens is necessary for normal pathfinding by R8 axons.

A temporal difference in transcription factor expression by R7 and R8 also contributes to their selection of the correct termination layers. The zinc finger protein

Sequoia (Seq) is transiently expressed first in R8 and subsequently in R7, during each cell's axonal growth phase [33•]. In *seq* mutants both axons terminate prematurely, R7 in the M3 layer and R8 in more superficial layers, while prolonged Seq expression in R8 drives it to the M6 layer [33•]. One target of Seq is the cell adhesion molecule N-cadherin (CadN), which stabilizes projections to the M6 layer formed by wild-type R7 cells and Seq-overexpressing R8 cells [8•,20,33•] (Figure 2). However, it is likely that the strong mistargeting phenotypes caused by defects in the expression of Seq and other transcription factors result from changes in multiple downstream adhesive and signaling molecules.

Homophilic adhesion molecules stabilize R7 and R8 axons in their appropriate target layers

Expression of specific homophilic adhesion molecules in response to cell fate-determining transcription factors can mediate the segregation of synapses into distinct layers. In the vertebrate retina, transmembrane immunoglobulin superfamily proteins of the Sidekick (Sdk) and Down's syndrome cell adhesion molecule (Dscam) families are expressed by non-overlapping subpopulations of interneurons and retinal ganglion cells and promote homophilic interactions that lead to synapse formation in specific sublaminae of the inner plexiform layer [34,35]. Similarly, R7 and R8 axons use homophilic adhesion molecules to stabilize their interactions with neurites of the appropriate target cell populations in the medulla.

The calcium-dependent Cadherins are an important class of homophilic adhesion molecules [36]. CadN is expressed in all photoreceptors and in their target cells in the brain, and plays a role in target selection by many of these cells [14,20,37]. Specific removal of CadN from R7 cells causes their axons to terminate prematurely in the M3 layer (Figure 1b and c) [20]. Loss of *CadN* from clones of cells in the medulla generates similar defects in R7 axonal projections, indicating that CadN acts both presynaptically and post-synaptically to mediate R7 target layer selection [38]. CadN is required in R7 for targeting to both its temporary and permanent layers [8•]. Although alternatively spliced isoforms encoding different transmembrane domains are used during these two developmental stages [39], diversity in the extracellular domain produced by alternative splicing is not essential to CadN function in R7 [8•]. In addition, a CadN transgene lacking the cytoplasmic domain is sufficient for initial targeting to the temporary layer and can also mediate targeting to the permanent layer to some extent [38]. CadN is thus thought to function primarily as a permissive adhesion molecule, rather than encoding recognition of diverse target cells in its isoforms or activating a cytoplasmic signaling pathway.

Flamingo (Fmi), a cadherin-related protein with 7 transmembrane domains, is required in the eye for R8 axons to reach the correct target layer [40] (Figure 1c). Since Fmi can mediate homophilic cell adhesion [41], and is expressed in both photoreceptors and medulla neurons [20,40], it may function by stabilizing contacts between R8 axons and their medulla target cells (Figure 2). However, removal of *fmi* from medulla neurons only very rarely causes premature R8 axon termination [7••].

R8 target layer selection is also regulated by another homophilic cell adhesion molecule, the leucine-rich repeat (LRR) protein Caps [32••]. In *caps* mutant clones in the eye, R8 axons often target either more proximal or more distal medulla layers than M3 [32••] (Figure 1c). Caps is likely to mediate R8 afferent-target interactions (Figure 2), since it specifically localizes to the projecting R8 axons and to their temporary and final target layers in the medulla [32••]; however, its role in the medulla neurons has not yet been examined. Strikingly, misexpression of *caps* in R7 cells forces their axons to terminate in the M3 layer [32••]. Similarly, misexpression of Sdk proteins in chick retinal neurons can redirect their neurites to a synaptic layer positive for the same Sdk [34,35]. It is not yet known whether Caps acts only as an extracellular adhesion molecule or whether its intracellular domain has an essential function, as it does during development of the trachea and wing [42,43].

Specific ligand–receptor interactions promote target layer selection

Synapse formation is thought to depend on heterophilic ligand–receptor interactions that lead to the assembly of intracellular effectors, a classic example being the interaction of presynaptic neurexins with postsynaptic neuroligins [44]. To date, the Anaplastic lymphoma kinase (Alk) and its secreted ligand Jelly belly (Jeb) constitute the only receptor-ligand pair that has been shown to control targeting of photoreceptor axons to the medulla [7••]. Impaired signaling from Jeb-expressing photoreceptors to Alk-positive medulla target neurons causes R8 axons to mistarget to either more distal or more proximal medulla layers, suggesting a failure to recognize the correct target cells (Figure 1c). Alk receptor tyrosine kinase signaling is required for normal expression of the cell adhesion molecules Dumbfounded/Kirre, Roughest/IrreC and Fmi in the M3 layer, indicating that Jeb released from incoming photoreceptor axons constitutes an anterograde signal that patterns the medulla to promote accurate target layer recognition [7••] (Figure 2).

The requirement for the transmembrane receptor Golden Goal (Gogo) in R8 for its recognition of the M3 layer suggests that signaling also occurs in the reverse direction. Gogo does not appear to act as a homophilic adhesion molecule, since it does not promote aggregation when expressed in cultured S2 cells, and its cytoplasmic domain is required for R8 targeting [6•]. Its essential extracellular Thrombospondin 1 and GOGO domains are thus likely to

bind a ligand that has not yet been identified [6•] (Figure 2). Driving the expression of Gogo at high levels throughout photoreceptor development leads all R8 axons to terminate in the M1 layer, perhaps owing to an excessive or abnormally sustained response to an attractive ligand normally expressed in this temporary layer, or to repulsion by a ligand present in the deeper layers [6•].

Transmembrane receptors are also required for normal innervation of the R7 target layer. Clones in the eye mutant for the *Insulin receptor* (*InR*) result in gaps in the projection to the M6 layer, although photoreceptor differentiation is normal [45] (Figure 1c). The InR controls R7 targeting through a direct interaction with the cytoplasmic adaptor protein Dreadlocks (Dock) [46], which regulates the cytoskeleton by recruiting the kinase Pak, rather than through the pathway used for cell growth control [45] (Figure 2). It is possible that *Drosophila* insulin-like peptides (DILPs) produced by a small cluster of neurosecretory cells and delivered through the circulatory system activate the InR in R7, although their lack of localized expression in the medulla makes them unlikely to generate an instructive signal. Interestingly, a homologue of the acid-labile subunit of the vertebrate Insulin-like growth factor-1 binding complex expressed in the DILP-producing cells controls motor neuron synaptic targeting, supporting a role for circulating DILPs in synapse formation [47,48].

Mutations in the genes encoding two receptor protein tyrosine phosphatases (RPTPs), LAR and PTP69D, cause R7 to mistarget to the M3 layer (Figure 1c). In *Lar* mutants, this defect has been shown to arise late in pupal development during targeting to the permanent layer (Figure 1b), and probably reflects a failure to stabilize synaptic contacts with the appropriate medulla neurons [8•,15,16,18,49•]. Since the phenotype of *Lar* mutant animals can be rescued by expressing LAR specifically in R7 [46], LAR is unlikely to act as a homophilic adhesion molecule. However, the ligands that regulate its activity and that of PTP69D in R7 have yet to be identified. The two RPTPs are unlikely to share a common ligand, since rescue experiments using chimeric transgenes show that the extracellular domain of LAR can substitute for that of PTP69D in R7 targeting, but not vice versa [15]. The heparan sulfate proteoglycans Dally-like (Dlp) and Syndecan (Syd) act as ligands for LAR in the context of motor axon pathfinding and synaptogenesis [50,51]. However, these ligands bind to the distal Ig domains of LAR, which are dispensable for LAR function in R7 targeting [49•]. In addition, loss of *syd* or *dlp* does not phenocopy the *Lar* mutant R7 targeting defect [52], suggesting the existence of a novel ligand that regulates the activity of LAR in R7.

Surprisingly, phosphatase activity is not required for LAR to promote R7 targeting, although structural features of its intracellular domain play an important role, suggesting that LAR assembles a complex of downstream effectors that contribute to synapse stability [49•]. These effectors are likely to include CadN and the scaffolding protein Liprin-α, which physically interact with LAR *in vivo* and show similar R7 targeting defects when mutated [20,53–55] (Figure 2). By contrast, PTP69D may act as a more conventional phosphatase [49•], although the substrates relevant to its function in R7 are unknown. Several serine-threonine kinases shown to play a role in medulla targeting [17] are candidates to act downstream of the receptors described in this section, but have not yet been linked to specific pathways.

Repulsive interactions maintain axons within separate columns

The R7 and R8 axons from one ommatidium project into the medulla together, contacting target cells in different layers but within the same column. The restriction of these axons to their own discrete column is known as tiling, a term originally used to describe mutual avoidance by dendrites from the same cell type that allows them to cover a target field without overlapping [56,57]. Tiling of R7 and R8 axons ensures proper assimilation and processing of color information, leading to normal visual behavior [19•].

Tiling of R8 axons appears to use some of the same molecules that mediate afferent–target interactions. *caps* mutant R8 axons often lose their columnar restriction and terminate in neighboring fascicles [32••]. Fmi is likewise necessary for spacing R8 axons in an organized array as they enter the medulla, since R8 axons projecting from *fmi* mutant eyes form abnormal bundles within the larval brain [40,58]. Gogo and Jeb/Alk signaling also contribute to normal tiling, perhaps by mediating repulsive interactions between R8 axons [6•,7••] (Figures 1 and 2). Alternatively, tiling defects in these mutants could be an indirect consequence of failure to form stable interactions with cells in their own column.

In R7 cells, the TGF-β family member Activin is thought to act as an autocrine signal that prevents the growth of R7 axons into other columns [19•]. Activin, which is expressed in both R7 and R8 cells but may be specifically activated in the M6 layer by the processing enzyme Tolkin [19•], binds to the serine/threonine kinase receptor Baboon (Babo) [59] in the R7 growth cone. Babo phosphorylates the transcription factor dSmad2, which is translocated to the nucleus by the adaptor protein Importin-α3 (Imp-α3) to regulate the expression of unknown target genes that constrain R7 growth cone extension [19•] (Figure 2). Disruption of Activin/Babo signaling or of retrograde transport in individual R7 cells results in the invasion of neighboring columns by mutant axons [19•] (Figure 1c). Interestingly, removal of neighboring wild-type R7 cells using a *sevenless* mutation [60]

dramatically enhances this invasion, suggesting that the Activin pathway is partially redundant with a second mechanism involving repulsion between neighboring R7 axons [19•].

This repulsion signal may be mediated by Turtle (Tutl), a member of the immunoglobulin superfamily, which appears to promote R7 tiling in parallel to the Activin pathway [61,62•]. Tutl acts both autonomously and non-autonomously; *tutl* mutant R7 axons can project laterally into neighboring columns, but *tutl* mutant R7 columns can also be invaded by their wild-type neighbors [62•] (Figure 1c). Tutl mediates homophilic interactions between cultured cells, and its cytoplasmic domain is dispensable for its function in limiting dendritic branching in larval sensory neurons, suggesting that it is capable of acting as a ligand or coreceptor [63]. Tutl could thus promote mutual repulsion between R7 axons either by activating an intracellular signaling pathway, or by interfering with the function of adhesion molecules such as CadN [62•] (Figure 2).

Conclusions

The simple and repetitive structure of the *Drosophila* visual system makes it a powerful model in which to investigate axon targeting. In contrast to the plastic response to neural activity characteristic of mammalian visual circuits [64], R7 and R8 target layer specificity appears to be genetically encoded by cell fate-specific transcription factors. The targets of these transcription factors include adhesion molecules with restricted expression patterns, such as Caps and Gogo. However, other molecules with specific effects on targeting, such as Jeb, CadN and LAR, are more broadly expressed. Their functional specificity may be due to distinct temporal patterns of expression [14,33•], or to a restricted distribution of ligands, coreceptors or downstream molecules. Surprisingly, some transmembrane proteins seem to contribute both to attractive interactions that promote target layer selection and to repulsive interactions required for tiling [6•,7••,32••,40], suggesting the possible existence of distinct sets of downstream factors. Our knowledge of the molecules that act in medulla neurons to promote accurate photoreceptor axon targeting is much less advanced, owing to the complexity of this population and the greater technical difficulties of genetic analysis. The transcription factors that control layer-specific expression of adhesion molecules, and the ligands that regulate several apparent receptors expressed on R7 and R8, have yet to be identified. Finally, the choice of synaptic partner cells is likely to be more complex than the choice of termination layer; although the majority of R7 synapses are formed in or close to the axon terminals, R8 synapses are more broadly distributed along the axon [12]. Future studies will fill in the missing components of the intricate combinatorial mechanism that assembles the visual circuitry.

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