**Title**

Untangling the wiring of the *Drosophila* visual system: developmental principles and molecular strategies

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**Running title**

Fruit fly visual system wiring

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**Abstract**

The assembly of neural circuits relies on the accurate establishment of connections between synaptic partners. Precise wiring results from responses that neurons elicit to environmental cues and cell–cell contact events during development. A common design principle in both invertebrate and vertebrate adult nervous systems is the orderly array of columnar and layered synaptic units of certain neuropils. This similarity is particularly striking in the visual system, both at the structural and cell-type levels. Given the powerful genetic approaches and tools available in *Drosophila*, the fly visual system has been extensively used to probe how specific wiring patterns are achieved during development. In this review, we cover the developmental principles and molecular strategies that govern the assembly of columnar units (lamina cartridges and medulla columns), the formation of layers, afferent specific layer selection, and synaptogenesis in *Drosophila*. The mechanisms include: sequential developmental steps that ensure coordinated assembly of synaptic partners; anterograde and autocrine signaling; interactions between cell-surface molecules, or secreted molecules and their receptors, that take place among neurons; and glia signaling to neurons.

**Key words:**

neural circuits, wiring specificity, synaptic partners, columns and layers, cell surface and secreted molecules

## Introduction

The neuroanatomist Santiago Ramón y Cajal was the first to notice the striking similarities between vertebrate retinas (Cajal, 1893). In his quest to understand the flow of information between neurons, he turned to the visual system of large flies expecting it to be simpler (Cajal, 1937). To his surprise, he discovered that the cellular diversity and complexity of the insect visual system parallels that of vertebrates. More importantly, he recognized the similarities between the neural circuits that underlie vision in vertebrates and flies (Cajal & Sanchez, 1915). Over the past few decades, structural, developmental and functional studies in these organisms have backed up Cajal's view (Kolodkin & Hiesinger, 2017; Sanes & Zipursky, 2010). This wealth of work suggests that conserved features are probably fundamental, and that knowledge obtained from one organism can provide insight into others.

Seminal work by Fischback and Dittrich (Fischbach & Dittrich, 1989) revealed that the smaller visual system of *Drosophila melanogaster* had the same complexity as that of larger flies. More importantly, it opened the door to the use of the visual system of this genetically amenable *Drosophila* species as a model of neural circuit assembly. While the functionality of circuits relies on accurate connectivity between neurons, this synaptic specificity is dependent on several previous steps, including: axons finding a path to the general target region (ganglion specificity); termination in a defined area in the target region (layer specificity); recognition of the specific synaptic partners among other neurons in the defined area; and finally, the formation of synapses (synaptogenesis). In fact, it is currently unclear whether recognition and synaptogenesis are two separable or intimately related events (see later in the text). Indeed, the

technical complexity of unequivocally assessing if presynaptic sites are apposed to corresponding postsynaptic partners in densely populated neuropils has so far limited the advance of our knowledge of this last step. In contrast, much insight has been gained into the other steps and in the past decade, particularly on how distinct neurons terminate and arborize in defined areas of the general target region, and the molecules that are involved in these processes. This is a developmental step that already limits the number of postsynaptic candidates that will be encountered. Given that the assembly of neural circuits results from cellular responses to environmental cues and cell–cell contact, cell-surface and secreted molecules have received much attention as mediators of these events.

In this review we summarize the developmental principles and molecular strategies known to date to govern the assembly steps of visual circuits in *Drosophila*.

## Visual system structural organization

### *Adult structure*

The retina of *Drosophila* is composed of approximately 750 units or ommatidia neatly organized in a lattice. Each ommatidium contains 8 photoreceptor neurons (R cells: R1-R8). These can be classified into three types: R1-R6 photoreceptors express the broad-spectrum opsin Rh1 and mediate motion detection; R7s express UV-sensitive opsins (Rh3 and Rh4) and R8s blue (Rh5) or green (Rh6) opsins. Three types of ommatidia are found depending on opsin expression in the R7 and R8 photoreceptors (Morante, Desplan & Celik, 2007). Ommatidia are classified into: *yellow* (70%), which contain

R7s and R8s expressing Rh4 and Rh6, respectively; and *pale* (30%), where R7s express Rh3 and R8s, Rh5. In the third type, Dorsal Rim Area (DRA) ommatidia, which are located in two rows in the dorsal edge of the retina and sense polarized light, both the R7 and R8 express Rh3. R cells relay the visual information into the optic lobe, the fly visual processing center, which consists of four ganglia (Fig. 1A): the lamina is the first relay station, just beneath it lies the medulla, which is followed by the lobula and the lobula plate. These last two ganglia form the lobula complex, from where processed visual inputs will be relayed to higher-order brain centers through different pathways (Mu, Ito, Bacon & Strausfeld, 2012; Otsuna & Kei, 2006; Otsuna, Shinomiya & Ito, 2014; Panser et al., 2016; Wu et al., 2016).

The spatial representation of the visual information in the visual system is morphologically supported by the presence of parallel columnar synaptic modules that process the information from discrete adjacent points of the visual field. These columnar modules are best studied in the lamina and the medulla (Fig. 1A). R cells collecting the information from a single point in space converge in one of these columnar modules. R1-R6 axons extend into the lamina where they form a discrete synaptic unit called a lamina cartridge with lamina neurons L1-L5, amacrine cells and centrifugal interneurons (Fig. 1B) (Meinertzhagen & Hansen, 1993; Meinertzhagen & O'Neil, 1991; Meinertzhagen & Sorra, 2001; Rivera-Alba et al., 2011). R7 and R8 axons extend past the lamina and project into the corresponding medulla column (Fig. 1C). In addition to R7 and R8 axons, medulla columns contain the axons of lamina neurons L1-L5, a diverse set of medulla neurons, and ascending higher order neurons amounting to a total of more than 80 neuronal types (Morante & Desplan, 2008; Takemura et al., 2008, 2013, 2015). Thus, the information from a point in space is

processed in its corresponding lamina cartridge and the associated medulla column. Hence, the repetitive columnar organization of the 750 registered lamina cartridges and medulla column sets covers the whole visual field in a retinotopic fashion thereby providing visual acuity.

Orthogonal to this columnar organization is the division of the medulla, lobula and lobula plate into parallel layers (Fig. 1A, C). This division arises from the unique arborization of neuronal processes of the distinct neurons in a column, where each neuron branches in one or more layers in specific patterns. This layered organization provides anatomically restricted regions that facilitate synaptic partner identification. Indeed, in many cases, presynaptic sites are enriched in terminals within specific layers, although synapses distributed along processes spanning different layers are observed in some cell types. This layered organization is the main structural basis for parallel processing, which allows the processing of distinct salient features at the same time. The best-studied example is the processing of color and motion, which are largely processed in separate streams and layers. Motion is first processed in the lamina by input from R1-R6 photoreceptors to lamina neurons, which in turn convey the information to the medulla according to their arborizations in specific medulla layers. Color detection arises from comparison of signals from R7 and R8 photoreceptors with different excitation spectra; these photoreceptors directly innervate specific layers of the medulla distinct from those receiving motion input. Electron Microscopy (EM) studies have structurally determined connectivity relationships among neurons within lamina cartridges and medulla columns. The work has revealed mini-circuits within these columnar modules, which in principle would allow for the processing of visual

information in different ways to extract salient features such as motion and color (Shinomiya et al., 2015; Takemura et al., 2011, 2013, 2017).

Visual information is thought to be integrated in space and time thanks to sets of neurons connecting adjacent columnar modules and layers. The fly visual system contains different neuronal types based on their morphology (Fig. 1A). These can be categorized into two main classes: the arborization of uni-columnar neurons is mostly limited to one column and thus they present a 1:1 ratio with columnar modules; in contrast, multi-columnar neurons possess processes in several columnar modules and thus are fewer in number than the columns (Fig. 1C). Inter-columnar connectivity can be supported by: 1) uni-columnar neurons whose processes extend laterally connecting neighboring columnar modules (e.g. L4 in the lamina, see below); and 2) multi-columnar neurons (e.g. Dm8, see below). Interlayer connectivity can arise from the arborizations of neurons projecting into various distinct layers (e.g. Tm20) or by neurons spanning several layers with synaptic inputs and/or outputs all along their main process (e.g. R8). These types of interactions between parallel relays, either between columns or between layers and mediated by interneurons, optimize the signal-to-noise ratio and mean that cells only a few synapses from the sensory input are already highly sensitive and selective to complex visual features.

All in all, the interconnected columnar and layer structure of the adult fly visual system covers the visual field and supports feature detection, respectively.

*Developmental establishment of the retinotopic map*

Two features of early eye development facilitate the innervation of the optic lobe in a spatially and temporally defined pattern (Fig. 2A) (Kulkarni, Ertekin, Lee & Hummel, 2016; Petrovic & Hummel, 2008; Roignant & Treisman, 2009). First, individual rows of ommatidia are recruited sequentially following a wave of cell division and differentiation beginning at the posterior end of the eye disc and sweeping anteriorly. The morphogenetic furrow, which results from the cytoskeletal changes cells undergo as they differentiate, is a moving boundary that separates the undifferentiated from differentiating tissue. Second, R cells in the same ommatidium extend axons into a single fascicle sequentially, following their differentiation order. R8 is the first photoreceptor to differentiate, followed in a pairwise fashion by R2/R5, R3/R4 and R1/6, and then finally by R7. The axonal fascicles of different ommatidia exiting the eye disc project into the optic lobe through the optic stalk. As axons exit this tightly packed axonal bundle, they must retain their positional information in the eye; that is, distribute evenly and recognize their targets along the anteroposterior and dorsoventral axes. Anterioposterior retinotopy is based on their time of differentiation in the eye disc, while dorsoventral innervation is regulated by asymmetrically expressed DWnt4 in the developing lamina and *iroquois* gene complex in the retina (Sato, Umetsu, Murakami, Yasugi & Tabata, 2006). R8 plays an important role in retinotopy as it is the first photoreceptor to innervate the optic lobe and acts as a guide to the rest of the R cells in the ommatidium. The non-classical cadherin Flamingo (Fmi) (Lee et al., 2003; Senti et al., 2003) and the Thrombospondin 1 domain and CUB domain transmembrane protein Golden goal (Gogo) (Tomasi, Hakeda-Suzuki, Ohler, Schleiffer & Suzuki, 2008) mediate axon–axon interactions between R8 processes as they exit the optic stalk in a bundle. These proteins facilitate the defasciculation of R8 cells from the bundle so that they enter the lamina evenly spaced, and continue into the medulla maintaining their

retinotopy. This ordered projection pattern is influenced by glial cells, which act as boundaries between developing ganglia and ensure the proper guidance of photoreceptors and other neurons into the medulla (Fan et al., 2005; Pappu et al., 2011; Tayler, Robixaux & Garrity, 2004).

R cells determine retinotopy between the retina and the lamina (Fig. 5A). This is achieved through R cell-dependent secretion of Hedgehog (Hh) and EGF (Spitz). Hh secretion from photoreceptors triggers terminal divisions in the lamina precursor cells field and their columnar assembly associated with each photoreceptor bundle according to their sequential innervation (Huang et al., 1998; Huang & Kunes, 1996; Sugie, Umetsu, Yasugi, Fischbach & Tabata, 2010; Umetsu, Murakami, Sato & Tabata, 2006). Meanwhile, EGF secretion from photoreceptors drives precursor differentiation into the five lamina neuron types in a precise spatio-temporal pattern (Huang, Shilo & Kunes, 1998). However, although Hh and EGF are concomitantly expressed, columnar assembly precedes differentiation. This delay is mediated by photoreceptors signaling through wrapping glia. This glial population ensheaths R cell axons in the optic stalk in response to photoreceptor Fibroblast Growth Factor (FGF) and they arrive in the optic lobe after the axons. EGF from photoreceptors activates EGF signaling in glia, which respond by secreting Insulin-like peptides, resulting in MAPK and InR signaling in lamina precursors (Fernandes, Chen, Rossi, Zipfel & Desplan, 2017). These signals, together with the autonomous requirement for a transcriptional network that regulates lamina neuron specification (Pineiro, Lopes & Casares, 2014), result in lamina neuron differentiation. In addition, during this time, R1-R6 photoreceptors terminate in the lamina plexus between two rows of glia, which provide an unidentified stop signal (Chotard, Leung & Salecker, 2005; Poeck, Fischer, Gunning, Zipursky & Salecker,

2001; Suh et al., 2002). Thus, different glial populations ensure that: 1) precursor columnar assembly and lamina neuron differentiation take place one after the other, so a reproducible number of precursors is present and lamina cartridges contain the complete set of lamina neurons; and 2) R1-R6 axons terminate in the medulla plexus and hence can associate with lamina neurons in the cartridges.

Our understanding of neurogenesis and neuronal differentiation in the optic lobe has advanced greatly in recent years (Fig. 2A) (Apitz & Salecker, 2015; Erclik et al., 2017; Neriec & Desplan, 2016; Suzuki & Sato, 2014); in particular, with regard to the medulla which develops from a crescent-shaped neuroepithelium called the outer proliferation center (OPC). A proneural wave transforms the OPC into neuroblasts. Neuroblasts undergo several rounds of asymmetric division to regenerate themselves and produce ganglion mother cells (GMC), which divide once more to generate postmitotic medulla neurons. Neuronal diversity in the medulla is generated though three mechanisms. First, the spatial patterning of the OPC generates three distinct dorsal regions determined by the specific expression of transcription factors and their ventral counterparts, thus giving a total of 6 spatially defined regions. Secondly, as neuroblasts are formed and age, they express a defined series of transcription factors (Li et al., 2013). The temporal progression of these factors is not affected by the dorsoventral patterning of the OPC. Finally, Notch-dependent binary cell fate gives distinct identity to the two daughter cells resulting from the division of a GMC (Li et al., 2013). Recent work provides data indicating that the temporal axis generates specific sets of neurons over time. It also shows that regional differences in the OPC confer spatial identities to neuroblasts with the same temporal identity. It appears that this positional information is erased from uni-columnar neuronal types, which are generated all along the dorsoventral axis; while

multi-columnar neurons are generated in specific regions and require spatial input (Erclik et al., 2017). Global and regional neuronal specification produces the proper cellular diversity and stoichiometry that supports the formation of medulla columns.

In contrast to the lamina, where photoreceptors induce the assembly and differentiation of lamina neurons, the establishment of retinotopy in the other neuropils requires further investigation. While photoreceptors do not seem to influence the generation of medulla neurons, mechanisms that match them to photoreceptors or lamina neurons and ensure the inclusion of the right set of medulla neurons in columns must exist. One such mechanism could be Eph/ephrin signaling, which regulates retinotopic mapping in vertebrates through graded expression of Eph receptors and ephrin ligands. The sole fly Eph receptor is expressed in a graded fashion in photoreceptors and the developing medulla (Dearborn, He, Kunes & Dai, 2002). In addition, disruption of this graded expression through various genetic means results in defects in both photoreceptor and medulla neuron axon guidance (Dearborn et al., 2002). Thus, this could be a pathway involved in mediating topographic recruitment of medulla neurons to their respective columns.

In summary, a matched set of ommatida, lamina cartridges and columns formed in a concerted way during development ensures the maintenance of the topographic representation of visual information in the optic lobe.

**Circuit assembly in the lamina**

*Neural superposition and cartridge formation*

Due to the curvature and structure of the compound eye, sensory input results in a complex wiring pattern in the lamina. While R7 and R8 cells are one on top of the other and collect information from the same point in space, the optical axes of R1-R6 photoreceptors in the same ommatidia point in different directions. One cell of each R1-R6 subtype located in 6 surrounding ommatidia sample the same visual point as the R7 and R8 cells in the central ommatidium. Thus, during a short time window in pupal development, R1-R6 axons from different ommatidia but "seeing" the same point in space converge onto the same lamina cartridge to make connections mostly with lamina neurons (Fig. 1B). However, since R1-R6 axons in one ommatidium project as one bundle onto the lamina, they must defasciculate to each reach a different cartridge (Fig. 2B). To this end, each R subtype properly orients its growth cone and extends different distances to its target cartridge, in some cases migrating over potential targets. Given that there are approximately 750 ommatidia in the fly eye, the sorting of some 4500 axons creates an intricate wiring scenario termed neural superposition (Kirschfeld, 1967). The biological significance of this wiring patter lies in the fact that superimposing multiple inputs from the same point in space into a single synaptic unit results in an enhancement of sensitivity without incurring an acuity penalty (Laughlin, Howard & Blakeslee, 1987).

Some aspects related to the sorting of axons have been characterized in detail (Meinertzhagen & Hansen, 1993). Among these are: the formation of an initial grid by lamina neurons that lie in the center of the cartridge and are synaptic partners of R1-R6 cells (see above); and molecular insight into how differential adhesion plays a key role in growth cone sorting (Chen & Clandinin, 2008; Schwabe, Neuert & Clandinin, 2013)

and target selection (Prakash et al., 2009; Prakash, Caldwell, Eberl & Clandinin, 2005). However, while previous studies suggested the possibility of simple developmental rules underlying this sorting process (Clandinin & Zipursky, 2000; Meinertzhagen, 1972; Meinertzhagen & Hansen, 1993), no rule set or algorithm sufficient to generate precise neural superposition has been formulated.

A recent study using high-resolution intra-vital time-lapse imaging of R1-R6 neurons and mathematical modeling defined 3 simple rules that specify target selection (Langen et al., 2015). A key aspect of that work was the observation of bipolar growth cones forming an anchor ("heel") at the arrival point and an extending "front", a feature that had never been observed in fixed tissue, and precise quantification of filopodia dynamics. Briefly, these rules are as follows: (1) Scaffolding rule: prospective target areas are defined by a grid of growth cone heels, which form a scaffold within the lamina, (2) Extension rule: growth cone fronts travel with remarkable constancy (with angle, speed and developmental time window being specific to the photoreceptor subtype) to arrive at the same time at the target area and (3) Stop rule: robustness of growth cones stopping at the correct target, despite overlapping with multiple wrong targets during their extension, is increased by the overlap of growth cone fronts in the target area. Importantly, the mathematical model based on these rules accurately predicts the targeting error rates that are naturally observed at the equator of the retina where the orientation of the ommatidia from the dorsal and ventral sides is a mirror image.

The proposed developmental algorithm serves as a framework to match the described molecular mechanisms described to the rules they execute. Indeed, some of the

molecular mechanisms identified so far are in accordance with the algorithm. For example, the scaffold may control polarization and hence the extension angle of R1-R6 cells within a bundle through R-cell interactions mediated by a redundant network of cadherins (Chen & Clandinin, 2008; Schwabe, Borycz, Meinertzhagen & Clandinin, 2014; Schwabe et al., 2013). Cell-type-specific levels of Fmi together with its subcellular localization, probably in heels, would generate specific adhesive interactions, most probably between different neighboring axons from the same bundles. The classical cadherin, N-cadherin (CadN), broadly localized in the growth cones might primarily mediate interactions between growth cones across bundles in addition to also functioning in intra-bundle interactions. The spatial constriction of axons and stereotyped intra- and inter-bundle neighbors generates stereotyped adhesive forces that shape and orient growth cones in a cell-type-specific manner. Live imaging together with selective perturbation of Fmi and CadN expression would be a way to validate the role of these molecules in the scaffold rule. With regard to axon extension, Fmi and CadN also seem to act in concert (Schwabe et al., 2013). However, it is unclear from the intravital imaging that extension is a separate step from orientation, or whether these adhesive interactions instruct extension itself (Langen et al., 2015). The mathematical modeling of the stop rule does not per se require interactions with lamina neurons in the target area, but appropriate target selection is more robust when combining coincidence detection of overlap of the R cell front and interactions of fronts with lamina neurons. Indeed, studies on CadN, together with tyrosine phosphatase receptor Lar and Liprin-alpha, suggest a role for this molecule in interactions between R cells and lamina neurons in the target region (Choe, Prakash, Bright & Clandinin, 2006; Prakash et al., 2009). Assessing the requirement of target cells would definitively reveal the relevance of lamina neurons for the stop rule.

Thus, the developmental algorithm that has been described, executed through afferent–afferent interactions and mediated by the redundant use of a small number of adhesion molecules with different expression and subcellular localization, could direct the formation of a complex wiring pattern.

*Cartridge interconnection*

Signal integration and modular connection is already observed in the lamina neuropil. R1-R6 photoreceptors primarily make connections with L1, L2 and L3. Meanwhile, L4 and L5 do not receive direct input from R cells, instead L2 establishes synaptic contacts with L4. Among the lamina output neurons, L4 neurons are unique in that they connect cartridges tiling the entire retina. Each L4 produces three primary dendrites: one that innervates its own cartridge; and two that project to the immediate dorso- and ventro-posterior cartridges (Meinertzhagen & O'Neil, 1991; Rivera-Alba et al., 2011; Strausfeld & Campos-Ortega, 1973; Takemura et al., 2011). Live imaging and genetic mosaics indicate that dendritic targeting of L4 is controlled by homophilic interactions mediated by Dscam proteins between L4 and at least some lamina neurons in the target cartridge (Tadros et al., 2016). In contrast to previous studies where Dscam1 and Dscam2 were considered to pattern circuits through homophilic contact-dependent repulsive interactions (Hattori, Millard, Wojtowicz & Zipursky, 2008; Lah, Li & Millard, 2014; Millard, Lu, Zipursky & Meinertzhagen, 2010), Tadros and colleagues describe an adhesive function for Dscam2 and Dscam4 in L4 dendrite patterning. A lack of either gene results in the formation of extra dendritic branches innervating additional cartridges. This phenotype is attributed to early defects in L4 dendrites, which fail to stabilize in the target cartridge and thus continue extending, most probably through

interactions with R cells. The fact that adult Dscam2/4 mutant L4s develop dendrites both in their target cartridges and in extra ones, suggests that Dscam2/4 could restrict filopodial exploration of target cartridges.

## Circuit assembly in the medulla

*Formation of columnar circuits in the medulla*

The establishment of medulla columns and layers, which takes place in parallel to cartridge formation, are processes that have mostly been addressed through the study of R7, R8 and lamina neurons. A relevant aspect of column formation is the restriction of neuronal processes to a single column; a process akin to tiling. In addition to serving the function of forming a complete, yet not overlapping receptive field, columnar restriction also involves the regulation of dendritic morphology and field size to match synaptic partners in a 3D space (Luo, McQueen, Shi, Lee & Ting, 2016). The mechanisms regulating columnar restriction described to date are: intrinsic autocrine signaling, anterograde signaling, homotypic interactions between neurons of the same type in neighboring columns, and heterotypic interactions between different types of neurons in the same column.

For example, R7 axons rely on two redundant intrinsic and extrinsic mechanisms to remain in their column (Ting et al., 2007). On the one hand, TGF-β signaling, mediated by autocrine dActivin (dAct) activation of Babo receptors, and Smad2 retrograde transport to the nucleus, prevents R7 axons from invading neighboring columns. It has been suggested that this signaling pathway blocks growth cone motility through

unknown downstream effectors (Fig. 3A). In parallel, repulsive interactions with neighboring R7 cells, which could be mediated by the homophilic interacting IgSF protein Turtle (Ferguson, Long, Cameron, Chang & Rao, 2009), contribute to the maintenance of R7 axons in their column.

Similarly to R7, L1 lamina neurons restrict their arborizations to a single column through interactions with L1 neurons in neighboring columns. The homophilic binding IgSF member Dscam2 mediates columnar restriction through lateral contact-dependent repulsive interactions (Fig. 3C) (Millard, Flanagan, Pappu, Wu & Zipursky, 2007). Consistent with the properties of a homophilic binding mechanism between neurons of the same type, wild-type L1 arbors display a non-autonomous phenotype of their processes invading only neighboring columns containing a *Dscam2*-mutant L1 neuron.

Adhesive interactions between neurons in the same column can act to locally limit arbors to a single column (Fig. 3D). Such a mechanism has been described for L5 arborizations at M2 and M5, where *CadN*-mutant L5 arbors invade adjacent columns (Nern, Zhu & Zipursky, 2008). CadN homophilic adhesive interactions between L5 and L2 neurons in the same column mediate L5 interstitial arbor extension into M2 and restriction to the column. In addition, similar to the phenotype observed in *CadN*-mutant L5 arbors, the absence of *CadN* in L2 results in branches of wild-type L5 of the same column contacting wild-type *CadN*-expressing L2 neurons in the neighboring columns. It is unclear how CadN regulates axonal tiling of L5 axonal processes in M5. It could be through a similar mechanism of loss of adhesion with other neuronal processes in that layer, where a likely candidate would be L1. Thus, even though L2 and L5 are not synaptic partners, this example highlights how afferent interactions can play

a key role in columnar restriction, and thus the local patterning of terminals within the target region.

Anterograde signaling has been proposed as an effective mechanism through which afferents could coordinate development of the target region. Photoreceptor induction of lamina development though Hh and EGF is one such example (Fig. 5A). In addition, the ligand Jelly belly (Jeb) primarily generated by photoreceptor axons modulates, through its receptor Anaplastic lymphoma kinase (Alk), the target region (Bazigou et al., 2007; M. Pecot et al., 2014; see later in the text and Fig. 5B). A recent study suggests that anterograde signaling is also an effective mechanism to shape the receptive field of synaptic partners (Fig. 3B) (Ting et al., 2014). In addition to the autocrine role of TGF-β signaling in R7 columnar restriction, dAct is also secreted from the R8 cell. In this case, dAct/TFG-β signaling regulates dendritic field patterning of the R8 postsynaptic partner Tm20 neuron, restricting its dendrites to a single column. However, the paracrine effect of this pathway is not specific to columnar restriction, but rather to the postsynaptic receptive field. dAct originating in the R7 process and acting in a paracrine fashion also shapes the dendritic field of postsynaptic Dm8 amacrine neurons, which receive inputs from some 16 R7 cells. In both cases, disruption of the dAct/TFG-β signaling results in the expansion of dendrites into additional adjacent columns.

*Layer formation in the medulla*

The development of layers is a temporal process with new layers emerging as new processes innervate the neuropil (Fig. 4). The stepwise nature of the medulla development suggests that early interactions between neurons actually happen in a much simpler environment than the final adult connectivity might indicate. In addition,

a staged development also allows for afferent target interactions that can facilitate the development of or encounter with synaptic partners in a local region.

Given that the complex connectivity in the medulla must arise from cellular interactions mediated by molecular handles, the transcriptional regulation of the cell type specific set of molecules involved in wiring is important (Tan et al., 2015). Indeed, transcriptional programs play a role in R7 and R8 layer selection. The sequential photoreceptor differentiation in the eye translates into the sequential innervation of the optic lobe (Fig. 2A), resulting in a two-step targeting process (Ting et al., 2005). Initially, R8 axons pause in a temporary layer, while R7 axons extend deeper in the medulla. In mid-pupal development, once the whole retinal complement of photoreceptors is positioned, the second step involves the synchronized active movement of all R8 axons from their temporary to their final target layer (Timofeev, Joly, Hadjieconomou, & Salecker, 2012; Ting et al., 2005) displaying discrete growth cone behaviors (Fig. 4) (Akin & Zipursky, 2016). These include: a phase of extension followed by stabilization to their targets and an elongation phase in which R8 axons increase in length in concert with the growing neuropil. Starting after the stabilization phase and overlapping with elongation, the growth cone transforms into a synaptic terminal (Akin & Zipursky, 2016). R7 growth cones already position themselves in the vicinity of and might make early contacts with their major postsynaptic partner upon entering the medulla (Ting et al., 2014). Their initial relatively deeper position in the medulla is maintained during the R8 second step by the intercalation of processes from lamina neurons and other neurons (Özel, Langen, Hassan & Hiesinger, 2015). This passive dislocation requires their continuous recognition of their targets and stabilization. This is achieved through specific filopodial types and their dynamic

patterns at different developmental steps of column restriction, layer separation and synaptogenesis (Özel et al., 2015), and the regulated intrinsic axonal growth of the R7 cell (Fig. 4) (Feoktistov & Herman, 2016; Kniss, Holbrook & Herman, 2013).

The Zn-finger transcription factor Sequoia (Seq) has been shown to regulate the segregation of R7 and R8 growth cones in the first targeting step during initial innervation of the medulla through relative temporal differences in *seq* expression (Petrovic & Hummel, 2008). A recent study goes on to propose that defects in this initial step condition selection of their target layers due to incorrect consolidation of growth cone positioning in the initial targeting, thus proposing a simple developmental algorithm that controls layer selection (Kulkarni et al., 2016).

Transcriptional control also plays a role in the second step of layer selection. Mutations in the NF-YC transcription factor revealed the role of the R8-specific Zn-finger transcription factor Senseless (Sens) in R8 layer selection, and indicated that a major factor in R7 layer selection is the NF-YC-dependent repression of Sens, and hence the R8 layer selection program in the R7 cell (Morey et al., 2008). Orthodenticle and Prospero transcription factors likely work in parallel to Sens and NF-YC in R8 and R7, respectively (Mencarelli & Pichaud, 2015; Morey et al., 2008). Direct evidence of how these genetic programs regulate the expression of which molecules is still lacking.

Nevertheless, genetic screens have identified several cell-surface molecules that can promote layer recognition or stabilization of contacts between R7 and R8, and target neurons. In the R8 photoreceptor, Gogo and Fmi play a role in layer selection that is independent of their earlier function in afferent–afferent interactions during the

formation of the retinotopic map. Given the broad expression of Fmi in all photoreceptor axons and in multiple layers, the current model proposes that Gogo is a functional associate of Fmi that adds specificity for R8 layer targeting (Hakeda-Suzuki et al., 2011). Gogo is required in the first step for R8 to adhere to the temporary layer. Later on, Fmi antagonizes Gogo function to release the R8 growth cone from the temporary layer and forms complexes with Gogo to promote M3 layer targeting. Meanwhile, it has been proposed that the Leucine Rich Repeat cell surface molecule Capricious (Caps) mediates consolidation of R8 growth cones in the temporary layer upon entering the medulla (Kulkarni et al., 2016). In addition to specific expression in R8 axons, Caps is present in neuronal processes populating several medulla layers, including the R8 target layer (Shinza-Kameda, Takasu, Sakurai, Hayashi & Nose, 2006). Loss-of-function phenotypes and misexpression experiments showing an instructive role for Caps, together with in vitro data supporting homophilic binding, suggested that Caps could mediate R8 layer recognition through homophilic adhesive afferent–target interactions (Shinza-Kameda et al., 2006). Recent additional genetic experiments suggest instead that the mechanism by which Caps plays a role in M3 targeting is through adhesive interactions with a yet unidentified heterophilic ligand (Berger-Muller et al., 2013). In addition, the receptor Frazzled (Fra) is expressed and cell-autonomously required in R8 photoreceptors to target the M3 layer (Timofeev et al., 2012); while its ligand Netrin (Net) is localized in the prospective R8 target layer (see below; Akin & Zipursky, 2016; Pecot et al., 2014; Timofeev et al., 2012). A recent study based on live imaging and detailed quantitative analysis of hundreds of wild-type and mutant R8 growth cones redefined the molecular function of Fra/Net, which was traditionally viewed as chemoattraction signaling. R8 growth cones mutant for *Fra* or in a *Net*-mutant background reach and recognize the target layer essentially as wild-type

R8 cells. Thus showing that in this context Fra and Net are required for the attachment of a single process extended from the R8 growth cones to the target layer. These findings favor the notion that rather than promoting directed outgrowth or target recognition, Fra mediates adhesion to neuronal processes or the extracellular matrix in the target layer (Akin & Zipursky, 2016). A recent study in vertebrates shows how *netrin-1* also acts locally promoting growth cone adhesion (Dominici et al., 2017).

R7 layer selection depends on the widely expressed cell adhesion molecule CadN, which regulates R7 targeting to what had been described as its temporary and final layer (Nern et al., 2005; Ting et al., 2005). *CadN*-mutant R7 cells mistarget the R8 layer, and the penetrance of the defect increases with the developmental time of the analysis, thereby suggesting retraction of terminals from an initial correct targeting (Ting et al., 2005). Similarly, removal of *CadN* from the target region also impairs R7 layer selection (Nern et al., 2005; Ting et al., 2005). Thus, it has been proposed that CadN mediates a permissive afferent–target interaction for layer-specific R7 targeting. This interaction appears to be solely adhesive in nature, because the cytoplasmic region of CadN, hence its signaling activity, is not required in R7 cells (Yonekura et al., 2006). Given that CadN is expressed in many neurons in the medulla, it has been difficult to understand how it contributes to R7 layer selection. A recent study using live imaging to analyze the filopodial dynamics of *CadN*-mutant R7 axons showed no initial targeting defects, indicating that CadN would not function as a targeting cue. Instead, a general slowdown of the filopodial dynamics was observed, which resulted in R7 cells not simply retracting but jumping back and forth between layers, including their correct target layer. Interestingly, *CadN*-mutant R7 cells still recognize their target layer but are unable to stabilize (Özel et al., 2015). These observations are consistent with reduced

adhesion, increased likelihood of destabilization of contacts even after targeting has concluded normally, and hence an increase in targeting defects in *CadN*-mutant axons over time. In addition, R7 axons also depend on tyrosine phosphatase receptor proteins PTP69D (Newsome, Asling & Dickson, 2000) and Lar (Clandinin et al., 2001; Maurel-Zaffran, Suzuki, Gahmon, Treisman & Dickson, 2001; Ting et al., 2005). Similar to *CadN* mutants, loss of function of these genes results in R7 mistargeting the R8 layer, but their mode of action is not fully understood. Lar phosphatase activity seems to be largely dispensable, while the wedge domain facilitates the recruitment of other components such as CadN and various liprins (Astigarraga, Hofmeyer, Farajian & Treisman, 2010; Hofmeyer, Maurel-Zaffran, Sink & Treisman, 2006; Hofmeyer & Treisman, 2009)

Molecules that regulate layer-specific targeting of the R7 and R8 axons tend to be widely expressed, yet they are required for targeting of these cells. Their dynamic expression through neuronal intrinsic transcriptional programs, together with distinct cellular interactions taking place during medulla development, could facilitate the formation of stable contacts only at particular developmental times. Indeed, this phenomenon can be observed for the requirement of *CadN* in lamina neurons (Nern et al., 2008). Similar to R7 and R8, their targeting of distinct medulla layers takes place in discrete targeting steps at specific developmental time points for each neuron. All lamina neurons, with the exception of L2, cell-autonomously require CadN for layer-specific targeting. CadN functions in each of them at different time points, and the requirements for layer choice differ between cell types but are not layer specific. For example, L3 requires CadN for M3 targeting, while R8 does not; L5 for interstitial branching into M2, while L2 does not; L1 for targeting to M5, while L5 does not (Nern

et al., 2008). Different mechanisms could mediate this strikingly widespread yet cell-type-specific CadN patterning function. Sorting by differential adhesion (Steinberg & Takeichi, 1994) could explain how high levels of CadN in L2 dendrites mediate L5 interstitial branch extension into M2. The mechanisms for other cell types could be the specific regulation of CadN activity through other cell-surface or cytoplasmic molecules. Alternatively, CadN cell-type-specific phenotypes could be explained by a temporally distinct requirement in each neuron of its adhesive function.

In addition, as our knowledge expands on how lamina neurons develop their projections into the medulla (Nern et al., 2008; Pecot et al., 2013), we are gaining a better understanding of the layer assembly process. For example, in L3 layer selection (Pecot et al., 2013) a first step involves L1, L3 and L5 growth cones initially targeting a common domain, even though they are destined for different layers (Fig. 5B). Then, L3 in particular goes through a sculpting process that results in its segregation into the M3 layer. Initial targeting relies on the cell-autonomous redundant functions of Sema1a repulsive interactions mediated by PlexA, with complementary expression in the target layer, and the adhesive function of CadN, in addition to other factors. The L3 sculpting phase depends on Sema1a through PlexA expressed in adjacent neurons. Thus, the current model suggests that the discrete final layer arrangement in the adult will emerge progressively through intercellular interactions between processes within the same or nearby domains.

The elegance and importance of these intercellular interactions is best exemplified in the coordinated assembly of the independent neural connections of the motion and color circuits, which is based on a cascade of axon-derived signals (Fig. 5B-C). Anterograde

signals from R1-R6, which relay input to L3, have been shown to regulate the survival of the L3 neuron (Pecot et al., 2014). This effect is mediated by the R1-R6 secretion of the Jeb ligand (Bazigou et al., 2007; Pecot et al., 2014) and its interaction with the Alk receptor (Bazigou et al., 2007) expressed in L3 dendrites (Fig. 5B) (Pecot et al., 2014). The L3 axon is initially positioned in between the R8 and R7 terminals (Nern et al., 2008), however, in the adult medulla, both neurons are located in the M3 layer. L3 neurons express the ligand Netrin, which is necessary and sufficient for R8 cells, which respond to this signal through the expression of the receptor Frazzled, to adhere to the M3 layer (Timofeev et al., 2012). Thus, while L3 and R8 are not synaptic partners, L3 survival is required for the correct targeting of R8 (Pecot et al., 2014). Interestingly, these two cells share several postsynaptic partners (Gao et al., 2008; Takemura et al., 2013) and thus their interdependence ensures a link between the motion and color pathways.

## Connectivity in the fly visual system

### *Approaches to visualize synapses*

Detailed maps of synaptic connectivity in the lamina and medulla neuropils have been generated by several Serial Section Electron Microscopy (SSEM) studies (Meinertzhagen & O'Neil, 1991; Rivera-Alba et al., 2011; S. Takemura et al., 2008, 2013, 2015). That work has provided information on the synaptic partners for a given neuron, and the number of synapses and their spatial location (i.e., synaptic profile and number of synapses per layer) as well as their directionality. However, in addition to the technical requirements to perform SSEM, which is being substituted by Focus Ion

Beam Scanning Electron Microscopy (FIB-SEM), analysis of datasets is extremely time consuming, even in the case of the latest semi-automated reconstruction pipelines. For example, the manual refinement of the SSEM dataset to reconstruct 379 neurons in the fly visual system (Takemura et al., 2013) required approximately 14,400 person-hours. Thus, at the moment, assessing variations of synaptic connections among cells of the same type and between different animals, or at different developmental time points, or in different mutant backgrounds or activity-dependent scenarios with electron microscopy is not feasible in most instances.

The limitations in the use of SSEM as an approach to detecting variations and defects in the connectivity map has driven researchers to develop alternative approaches to visualizing synapses using light microscopy. Given the elevated number of cell types and density of processes in neuropils, those approaches aim to effectively visualize synapses in sets of desired neurons. Two main types of strategies have been used. One is based on the targeted expression of tagged synaptic proteins to label presynaptic or postsynaptic structures. The most commonly used synaptic protein is Bruchpilot (Brp) a component of the T-bar presynaptic structure, typical of synapses in the visual system and the element used in transmission electron microscopy to identify an active presynaptic zone. Using confocal microscopy, Brp is detected as fluorescent puncta when stained with Brp antibody, and each puctum correlates with one active presynaptic zone (Fouquet et al., 2009; Hamanaka & Meinertzhagen, 2010). The expression of tagged Brp, either through a heterologous promoter (the Gal4/UAS system: UAS-Brp short fluorescent constructs) (Fouquet et al., 2009; Górska-Andrzejak et al., 2013) or through its endogenous regulatory sequence (BAC recombineering: Synaptic Tagging with Recombination (STaR)) (Chen et al., 2014), identifies the same number of

synapses as those identified in EM studies, as well as the spatial distribution of the presynaptic sites, which provides a synaptic profile (Chen et al., 2014; Sugie et al., 2015). Thus, this type of approach can provide insight into the dynamics of synapse formation and the molecular mechanisms that regulate the assembly or stability of synapses in the visual system (Chen et al., 2014; Sugie et al., 2015).

A clearer validation of presynaptic puncta could in principle come from simultaneous detection of postsynaptic densities. Following this line of thought, an alternative approach to identifying synapses is the visualization of protein interactions across the synaptic cleft. In GFP Reconstitution Across Synaptic Partners (GRASP), these interactions are observed through the reconstitution of GFP fluorescence (Feinberg et al., 2008; Gordon & Scott, 2009). Synaptic GRASP relies on the expression of at least one of the split GFP domains under a synaptic protein in one of the cell types under study, and the other split GFP domain, synaptically localized or not, in the other cell type. A recent modification allows for single-cell GRASP, and also includes a Brp-mCherry construct in one of the cell types, confirming the synaptic nature of the GRASP signal and the directionality of the connection (Karuppudurai et al., 2014). In addition, a vesicle-tethered split GFP version (syb-spGFP1:10) can be used as an activity-dependent signal (Frank, Jouandet, Kearney, Macpherson & Gallio, 2015; Karuppudurai et al., 2014; Macpherson et al., 2015). Hence, GRASP has the potential to identify connectivity changes between synaptic partners. In addition, GRASP can allow us to identify postsynaptic partners through the expression of one of the split GFP components with drivers for candidate neuronal populations of the region (Lin et al., 2016).

Given that overexpression of tagged synaptic proteins and GRASP components could alter the number of synapses between particular combinations of neurons, EM confirmation stands as the definitive proof of connectivity.

*Conversion of growth cones to synaptic terminals*

Morphological changes in growth cones have been described in detail in both photoreceptors and lamina neurons as they transition to synaptic terminals (Akin & Zipursky, 2016; Chen et al., 2014; Clandinin & Zipursky, 2002; Nern et al., 2008; Özel et al., 2015; Ting et al., 2005). The use of Brp has allowed to follow the formation of presynaptic structures in photoreceptors during development, both in fixed and live samples (Akin & Zipursky, 2016; Chen et al., 2014), and has provided a correlation between the observed morphological changes and synapse formation. The time course of presynaptic development is highly similar for all three classes of photoreceptor neurons. The formation of presynaptic sites initiates, for all these neurons, in the same time window, which coincides with the extension of axons marking the transition from growth cones to synaptic terminals. The addition of presynaptic sites continues to adulthood, after layer selection is completed (Chen et al., 2014). Interestingly, a recent study reported that different types of astrocyte-like glia populate the medulla neuropil and branch within particular layers and columns at the time of synaptogenesis (Richier, Vijandi, Mackensen, & Salecker, 2017). This observation raises the possibility that neuron–glia interactions influence the assembly of synapses.

Gene expression changes during presynaptic differentiation in photoreceptors have revealed prominent and dynamic changes in associated cell-surface molecules consistent with the morphological transformation of growth cones to synaptic terminals

(Zhang, Tan, Pellegrini, Zipursky & McEwen, 2016). Expression changes in genes encoding presynaptic proteins are modest. Most of these genes are already expressed at moderate levels at early stages, well before synapse formation. However, the length of their 3'UTRs increased along time while enzymes regulating 3'UTR cleavage and polyadenylation site selection were downregulated. Together with the presence of enriched RNA binding protein motifs for proteins known to regulate mRNA localization and translation in the extended 3'UTRs, these findings reinforce the idea that posttranscriptional mechanisms play an important role in presynaptic differentiation.

*Molecular determinants of synaptic arrangements*

While synapses are typically depicted as a presynaptic terminal associated with a single postsynaptic site, other arrangements have been described. A presynaptic side abutting multiple postsynaptic structures is a common finding, both in the vertebrate retina and insect visual systems and brains (Strausfeld & Meinertzhagen, 1998). A synapse with two postsynaptic partners is called a dyad; while one with three is a triad; and with four it is a tetrad. While it is unclear what the function of these divergent synaptic arrangements is, some ideas have been put forward (Meinertzhagen & Sorra, 2001). One possible explanation for this organization is metabolic economy, whereby one presynaptic site influences several postsynaptic partners. In addition, in systems with a relative low number of cell types, multiple-contact synapses could contribute to increasing synaptic complexity (i.e., progressing from an ancestral dyadic condition to tetrads through evolution) without the need to create new cell types. Another predictable function of multiple-contact synaptic structures is the strict temporal synchronization of the inputs to all the postsynaptic partners. Recently, a novel synaptic arrangement was

described for the first time in insects (Martin-Peña et al., 2014). It has been termed a coincident synapse and it consists of two independent cells with active zones at the same level on the same postsynaptic element. This results in active zones that are one in front of the other, maximizing the possibility of mutual influence. It has been hypothesized that this arrangement, although found in low percentages, could be the ultrastructural basis for coincidence detection and may mediate signal integration. Coincident synapses have been observed in the olfactory system and it would be interesting to assess their presence in the visual system.

The lamina is one of the most completely defined networks of synaptic circuits in any visual system (Meinertzhagen & O'Neil, 1991; Rivera-Alba et al., 2011). The small dimension of the cartridges and limited number of synaptic members has allowed researchers to use EM approaches to study the composition of synaptic arrangements and molecules shaping them. Despite the reduced number of cell types in the lamina, the repertoire of synapses that these cells form are richly diverse: the lamina contains many dyads of different classes and many tetrads of the same class. R1-R6 cell tetrad synapses with lamina neuron dendrites contain an invariable set of postsynaptic elements: always one L1 and one L2 element and two additional cells, typically amacrine cells or epithelial glia (Meinertzhagen & O'Neil, 1991). In this developmental context, Dscam1 and Dscam2 homophilic repulsive proteins act redundantly to ensure that L1-L1 or L2-L2 complexes do not form in the tetrad (Millard et al., 2010). Given that dendrites of L1 and L2 randomly associate at nascent tetrads, it has been suggested that Dscam1/2 regulate synaptic specificity by excluding inappropriate conformations in multiple contact synapses.

Similarly, the L4 synaptic network includes reciprocal synapses between L4 dendrites from neighboring cartridges and dendrites of L2. These synapses are almost eliminated in the absence of the cell adhesion molecules Kirre, a member of the irre cell recognition module (IRM) (Fischbach et al., 2009). In particular, all tetrad and triad synapses containing more than one L4 are absent. A model in which Kirre is required to stabilize synapses containing a pair of L4 neurons fits with this finding (Lüthy et al., 2014).

*Synaptic partner selection and synaptogenesis dependence*

Standing questions in the field include whether synapse formation is cell-autonomously regulated, and whether it depends on the recognition of synaptic partners.

Studies carried out in the lamina suggest that formation of the correct average number of synapses in R1-R6 (approximately 50 synapses) is independent of their correct sorting into cartridges and the specific mutations causing cartridge missorting (Hiesinger et al., 2006). Thus, R cells in a cartridge always present the same number of synapses, independently of the number of R cells in that cartridge. Given that the area of the main postsynaptic partners (L1, L2) does not increase proportionally to the presence of extra R1-R6 cells in missorted cartridges, Hiesinger and colleagues interpret the presence of the average number of synapses in photoreceptors as a result of an autonomous photoreceptor cell program. Still, given that the complementary experiment (excess of postsynaptic partners in unaltered cartridge composition) could not be assessed, it has not been possible to fully rule out a postsynaptic influence (Hiesinger et al., 2006).

In addition, R1-R6 photoreceptors that aberrantly extend into the medulla through overexpression of the transcription factor Runt make synaptic connections in this neuropil (Edwards & Meinertzhagen, 2009) in addition to their wild-type connections in the lamina. The most striking observation of this study was the synaptic composition of synapses in the medulla, which, as in the lamina, were tetrads. This is in contrast to R7 and R8 synapses that mostly form triads and occasional tetrads (Takemura et al., 2008). While it remains possible that misguided R1-R6 make connections with axons of their natural synaptic partners (L1/L2/L3) when they project as deep as to M5 (L1 arborizations are at M1 and M5; L2 in M2; and L3 in M3), synapses formed at M6 must be formed with novel partners. In addition, even though the glia that invaginates capitate projections (sites of vesicle endocytosis) in the medulla is different from the glia at synaptic sites in the lamina, capitate projection size and shape in the medulla was characteristic of R1-R6 synapses, and different from capitate projections in R7 and R8. Thus, all together, this supports cell-autonomous determination of synaptic architecture by presynaptic terminals.

The relationship between axon targeting and synaptogenesis has also been addressed in the R7 photoreceptor by misdirecting its axons to incorrect layers using the overexpression of cell-surface molecules (Berger-Muller et al., 2013). When R7 axons mistargeted the R8-recipient layer by means of Caps misexpression, the R7 cells maintained an R7-like synaptic profile. Interestingly, R7 axon misguidance through overexpression of Fmi and Gogo, resulted in a profile similar to that of R8 cells, indicating that these molecules could instruct R7 cells to make connections with R8 postsynaptic partners. It also suggests that these molecules might control synapse formation at the subcellular level in R8. When R7 axons were redirected to a more

superficial medulla layer using Unc5, the number of presynaptic puncta was reduced. While the nature of postsynaptic partners in these retargeting experiments would have to be confirmed by EM, these results indicate that cell-surface molecules are able to dictate synapse loci by changing the axon terminal identity in a partially cell-autonomous manner, but that presynapse formation at specific sites also requires complex interactions between presynaptic and postsynaptic elements.

In the case of R8, growth cones that were mispositioned at the temporary R7 layer through a pulse of *seq* misexpression, terminate, as wild R7 cells do, in the adult M6 layer (Kulkarni et al., 2016). Similar to R7 cells, R8 growth cones misdirected to the R7 temporary layer are in close proximity to R7 Dm8 synaptic partners. Interestingly, a GRASP signal can be detected in the adult between M6 mispositioned R8 cells and Dm8 cells, indicating that R8 cells can also recognize Dm8 processes and form synaptic contacts (Kulkarni et al., 2016). It also suggests that the selection of synaptic partners is dependent on the initial positioning of growth cones. Although this observation was made with an activity-dependent modified GFP reconstitution method (syb-GRASP; Frank, Jouandet, Kearney, Macpherson & Gallio, 2015; Karuppudurai et al., 2014) and *seq* misexpression does not seem to affect R8 differentiation, this technique does not reveal the synaptic profile of the mistargeted R8 cells.

The connectivity map is an invaluable starting point to study the molecular mechanisms and relationship behind synaptic partner selection and synaptogenesis. For instance, the medulla connectome has contributed to our understanding of synaptic connectivity through the identification of Ig superfamily ligand and receptor pairs expressed in synaptic partners (Tan et al., 2015). Using RNAseq and protein tagging Tan and

colleagues demonstrated that 21 paralogs of the Dpr family are expressed in unique combinations in R7, R8 and lamina neurons with different layer-specific synaptic connections. Expression analysis of their cognate binding partners, the 9 members of the Dpr interacting protein (DIP) family (Özkan et al., 2013), revealed complementary layer-specific expression in the medulla, which suggests that there are interactions between neurons expressing Dprs and those expressing DIPs in the same layer. Through coexpression analysis and correlation to the connectivity map, this work identified 10 examples of neurons expressing DIPs as a subset of the synaptic partners of lamina neurons expressing Dprs. In addition, an accompanying study (Carrillo et al., 2015) focusing on the Dpr 11-DIP γ interacting pair identified paired expression in *yellow* R7-Dm8 and T4/T5 medulla neurons-lobula plate tangential cell synaptic pairs. The report of 12 examples, between the two studies, suggests that Dpr-DIP interactions might regulate connectivity between the neurons expressing them. While some abnormalities in *yellow* R7 terminals have been reported in mutants for both Dpr 11 and DIP γ (Carrillo et al., 2015), the function of this particular Dpr-DIP pair is still unclear. Additional studies of this and other Dpr-DIP pairs are required to clarify their roles in wiring.

**Conclusions and future directions**

Besides the striking similarities between the fly and vertebrate visual systems, the power of current genetic methods has made *Drosophila* a successful model in the study of wiring specificity. Genetic screens for visual behavior have identified key determinants in photoreceptor axon targeting (Clandinin et al., 2001; Lee, Herman,

Clandinin, Lee & Zipursky, 2001) and sophisticated genetic techniques have enabled studies at the single-cell level both in wild-type and mutant conditions. Nevertheless, the commitment of the community working with the fly to develop new tools and approaches to unravel the mechanisms underlying wiring specificity has been decisive in this past decade to expand studies beyond photoreceptors. Among these contributions are the efforts of several groups at the Janelia Research Campus. Their work includes the generation of collections of fly lines and optimized tools that are aimed at labeling specific neuronal cell types in the optic lobe (Jenett et al., 2012; Nern, Pfeiffer & Rubin, 2015; Pfeiffer et al., 2008, 2010; Pfeiffer, Truman & Rubin, 2012), the EM reconstruction of 7 medulla columns and the generation of the medulla connectome (Takemura et al., 2008, 2013, 2015). These reagents and other tools have facilitated the study of other cell types in the visual system and expanded studies to postsynaptic pairs (Gao et al., 2008; Millard et al., 2007; Tan et al., 2015)

Through the study of specific genes and phenotypes of distinct neuronal types, the work presented here has outlined general principles and developmental strategies underlying the wiring of the fly visual system. For one, evidences of coordinated assembly is present in different parts of the visual system. Starting from spatio-temporal differentiation of photoreceptors, which results in lamina neuron induction to ensure proper formation of cartridges, to an as-yet-unknown coordination in the medulla to ensure that a whole set of medulla neurons associates with photoreceptors and lamina neurons in each column. In this way retinotopy is maintained throughout the visual system. Stepwise assembly is a feature that is also present in different regions. It is an essential aspect in the assembly of cartridges with the segregation of columnar organization of lamina precursors and their posterior differentiation into lamina

neurons. Similarly, the formation of layers is a developmental process where the adult structure emerges through a series of intercellular interactions. The coordinated stepwise development is mediated by sequential axon-derived signals. This signaling, mostly anterograde, regulates aspects such as the postsynaptic partner number matching in the lamina cartridges, trophic support of neurons and modulation of postsynaptic neuron dendritic fields in the medulla. These strategies suggest that the developmental environment in which circuits assemble in a sequential fashion is much simpler than it is in the adult structure. This stepwise assembly facilitates interactions with limited numbers of neurons and results in early recruitment or dismissal of synaptic partners.

From the molecular standpoint, most of the molecules identified in genetic screens tend to be broadly expressed (like CadN) and act in a permissive fashion, while they mediate the targeting of different afferents at different stages. This is in contrast to the classical view suggested by the chemoaffinity hypothesis (Sperry, 1963), in which neurons are molecularly specified to connect to their synaptic partners in a lock-and-key mechanism. The birth order and location of neurons together with the stepwise assembly of circuits limits the range of interaction options that a neuron encounters, and thus synaptic specificity might be mediated by the repeated use of a small set of molecules that could provide a spatio-temporal code for wiring specificity. Recent studies are challenging the idea that these molecules act as a code, and put forward the view that developmental algorithms based on pattern formation rules could explain wiring of neural circuits without the need for a code (Hassan & Hiesinger, 2015). This idea is based on the identification of “non-code” cell-intrinsic functions of some cell-surface molecules. That is, if the axon-targeting or branching choice they mediate does not involve a target cell, they do not function as a code. Interestingly, a fair number of

target-independent wiring mechanisms have emerged (Petrovic & Schmucker, 2015). Notable examples in the fly visual system are: homotypic repulsive interactions, heterotypic adhesive interactions, growth factor-dependent autocrine signaling to ensure proper spacing, and columnar assembly in the medulla; also differential adhesion between afferents to drive growth-cone polarization and target specificity in the lamina.

The study of mutants aims to perturb parts of the system with the hope of revealing gene function and uncovering rules. However, given the relevance of wiring, redundancy or compensatory responses to minimize the absence of one of the parts can confound the interpretation of the phenotypes observed, making it difficult to identify these rules. The recent development of live imaging protocols for photoreceptors is making important contributions in this direction. The observation of live growth cones during development is revealing cytoskeletal behavior both in wild-type and mutant conditions that was previously missed in fixed preparations. This approach is also revealing pattern formation rules, and helping to reinterpret previous findings and reformulate the conclusions drawn from previous studies (Akin & Zipursky, 2016; Langen et al., 2015; Özel et al., 2015). In addition, in vivo imaging clears the path to careful and detailed studies that could address remaining questions such as: whether genes with seemingly similar targeting phenotypes observed in fixed samples affect the same discrete developmental steps; if those genes differentially impinge on cytoskeletal behavior and what the changes they cause are; and how seemingly partially redundant functions converge in the regulation of cytoskeletal behavior. In addition, little is known about the specific molecular mechanisms and signaling pathways that link distinct cell-surface molecules to cytoskeleton changes.

The knowledge we have gained as well as the new tools and technical developments available open exciting new avenues. The identification of synaptic pairs and reagents that could be used to study the development of postsynaptic partners is an exciting field. This is already permitting the study of the mechanisms that regulate dendrite development and their receptive fields (Luo et al., 2016; Ting et al., 2014). This type of studies, coupled with the visualization of synapses with light microscopy, will open the door to the study of synaptic specificity, synaptogenesis between partners and the exploration of synaptic plasticity in different scenarios. In addition, an important future application of variations to label active synapses, such as activity dependent-GRASP (Frank et al., 2015; Karuppudurai et al., 2014) and activity-dependent, multi-color fluorescence reconstitution across synapses (X-RASP) (Macpherson et al., 2015), is to detect functional synaptic partners in visual system regions, such as the lobula (Lin et al., 2016), for which no connectome has yet been generated. This will be the first step towards expanding our knowledge of the circuits that regulate distinct visual features and trace them from the medulla to higher visual centers in the brain. In addition, little is known about the unique components of circuits downstream of pale, yellow or dorsal rim ommatidia, and if or how they maintain retinotopy.

It is well established that to fully understand the mechanisms regulating wiring and neural circuit function, in addition to neuron- neuron interactions, it is also important to take into account glial cell biology and glia-neuron interactions (Freeman, 2015). Indeed, the fly visual system contains many different glial types (Edwards & Meinertzhagen, 2010; Hartenstein, 2011) and some have been shown to play important roles in different aspects of circuit assembly. We have aimed to reflect this intimate relationship by including the diverse glia functions throughout the text, rather than in a

separate section. Glial contributions to neural circuit assembly include: the relay of signals to induce neuronal differentiation (i.e., lamina neurons) (Fernandes et al., 2017); ganglion specificity (i.e., R1-R6 photoreceptors terminating in the lamina); and the formation of boundaries between brain regions, facilitating axon guidance paths (i.e., R7 and R8 axon guidance to the medulla) (for a review of glial roles in the visual system, see Chotard & Salecker, 2008). Also of interest is the columnar- and layer-specific arborization of astrocyte-like glia in the medulla neuropil at the time of synaptogenesis, underscoring a possible role of glia in this process (Richier et al., 2017). Recent studies have elucidated: how differentiation of distinct glial populations is orchestrated (Bauke, Sasse, Matzat & Klambt, 2015; Chen et al., 2016; Sasse, Neuert & Klämbt, 2015; Suzuki, Takayama & Sato, 2016); the progenitor origin and migration of different glial subpopulations (Chen et al., 2016; Omoto, Lovick & Hartenstein, 2016); and which drivers label and genetically manipulate distinct glial populations during development and in the adult (Edwards & Meinertzhagen, 2010; Edwards, Nuschke, Nern & Meinertzhagen, 2012; Kremer, Jung, Batelli, Rubin & Gaul, 2017). It is expected that this information, together with the appropriate genetic tools, will allow us to further test the roles of glia in wiring and circuit function; especially in the medulla, where glial function remains unexplored.

In summary, in addition to open questions remaining, novel ones prompted by the progress made in the past decade will lead to further understanding of the mechanisms that regulate wiring. Moreover, given the similarities between the fly and the vertebrate visual systems (Kolodkin & Hiesinger, 2017; Sanes & Zipursky, 2010), it is expected that findings in both systems will continue to contribute in a complementary way to the

identification of conserved developmental principles regulating the wiring of neural circuits.

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**Figure legends**

**Figure 1. Adult structure of the fly visual system. (A)** Illustration representing the main different types of neurons in the optic lobe and their synaptic organization and relationship. R1-R6 photoreceptors terminate in the lamina neuropil and relay motion information to lamina neurons (L1-5) in synaptic units called lamina cartridges. This information is in turn relayed to the medulla by the axonal projections of lamina neurons, each with its unique arborizations in specific medulla layers. R7 and R8 photoreceptors send color information directly to medulla layers M6 and M3, respectively. Motion and color information converges in medulla columns where it is further processed. A registered set of ommatidia, cartridges and columns ensure the coverage of the visual field in a retinotopic fashion. Different types of neurons in the medulla process the visual information. Among these are intrinsic multi-columnar medulla neurons such as the Dm and Pm types, with arborizations spanning more than one column in the distal (M1-M6) or proximal (M7-M10) medulla, respectively. The medulla intrinsic neurons (Mi) are uni-columnar and arborize in various layers in the medulla. The Tm types send processed information from the medulla to the lobula plate; while the TmY types, in addition, project to the lobula plate. Specific members of these

classes of neurons arborize in distinct layers of these neuropils. **(B)** Detailed schematic of a lamina cartridge, which is the synaptic unit that pools visual information of one point in space. Due to the curvature of the eye, the R cells that "see" the same point in space are located in different ommatidia; they converge in the central cartridge, which contains the axons of R7 and R8 "seeing" that same point in space. R1-R6 neurons make connections with L1, L2 and L3 lamina neurons. **(C)** Representation of the relative positions of R7 and R8 terminals and lamina neuron arborizations in distinct layers, shown separately in panel (A), in one column. Colors correspond to the cell-type specific color code in (A). Medulla columns contain some 80 different types of neurons; for simplicity only 7 are superimposed here. Among medulla neurons, many are uni-columnar, such as Tm20, with one neuron present in each of the columns. Other neurons, such as Dm8, are present in fewer numbers than columns. However, with arborizations spanning multiple columns, they cover the visual field.

**Figure 2. Innervation of the optic lobe.** The R cell and lamina neuron (Ln) color code is the same as in Fig. 1. **(A)** Illustration of the late 3$^{rd}$ instar larval eye disc and optic lobe. Medulla and lamina neurons are generated from the OPC neuroepithelium (NE). On its lateral side, after the lamina furrow (LF) lamina precursor cells (LPC) are generated. On its medial side, medulla neurons (Mn) originate from a proneural wave that transforms the NE into neuroblasts (Nb). Another profileration center, the distal Inner Proliferation Center (dIPC) generates distal cells and lobula plate neurons not depicted here. Sequential differentiation of ommatidial rows and sequential R cell recruitment into developing ommatidia results in sequential innervation of the optic lobe. This creates a wave of differentiation of lamina neurons (Ln, stacked circles in shades of green) and termination of R1-R6 in the lamina plexus. The wedge shape of

the developing medulla reflects the sequential innervation by R8 and R7 neurons. The wide edge of the wedge shows R8 and R7 axons of the first differentiated ommatidia (older); while the thin end so far only contains R8 axons from recently differentiated ommatidia (younger). Although not depicted for simplicity reasons, lamina neuron axons will extend into the developing medulla in close association with R7 and R8 axons in each column, and project between the R8 and R7 terminals. Medulla neurons originate from the terminal division of ganglion mother cells (GMC). As Nb age, their progeny expresses distinct transcription factors giving rise to different types of medulla neurons and these will integrate into developing columns. **(B)** Detailed schematic of the R1-R6 selection of their respective target cartridge. Photoreceptors from a single ommatidia project to the lamina in a bundle. Starting at 20 hours after puparium formation (APF), each specific R1-R6 subtype must defasciculate, orient and extend to its specific target cartridge in a stereotyped fashion. This results in the formation of lamina cartridges pooling information from R cells "seeing" the same point in space.

**Figure 3. Molecular mechanisms that mediate columnar restriction.** Depiction of the columnar organization of specific cell types in the wild-type medulla (left side of the panel), and the effects in mutant situations (right side of the panel). Mutant neurons are outlined in red. **(A)** It is easy to envision that cell intrinsic mechanisms regulating axon growth could be part of the mechanisms ensuring columnar restriction. This is one of the mechanisms that mediates R7 axon columnar restriction. R7 axons mutant for members of the TFG-β signaling pathway such as the ligand (dAct), the receptor (Babo) or downstream effectors (Smad2), fail to respond in an autocrine fashion to the pathway and extend laterally into neighboring columns. Interestingly, while R8 cells also express dAct, mutations do not affect columnar restriction of this cell. **(B)** Interestingly, dAct

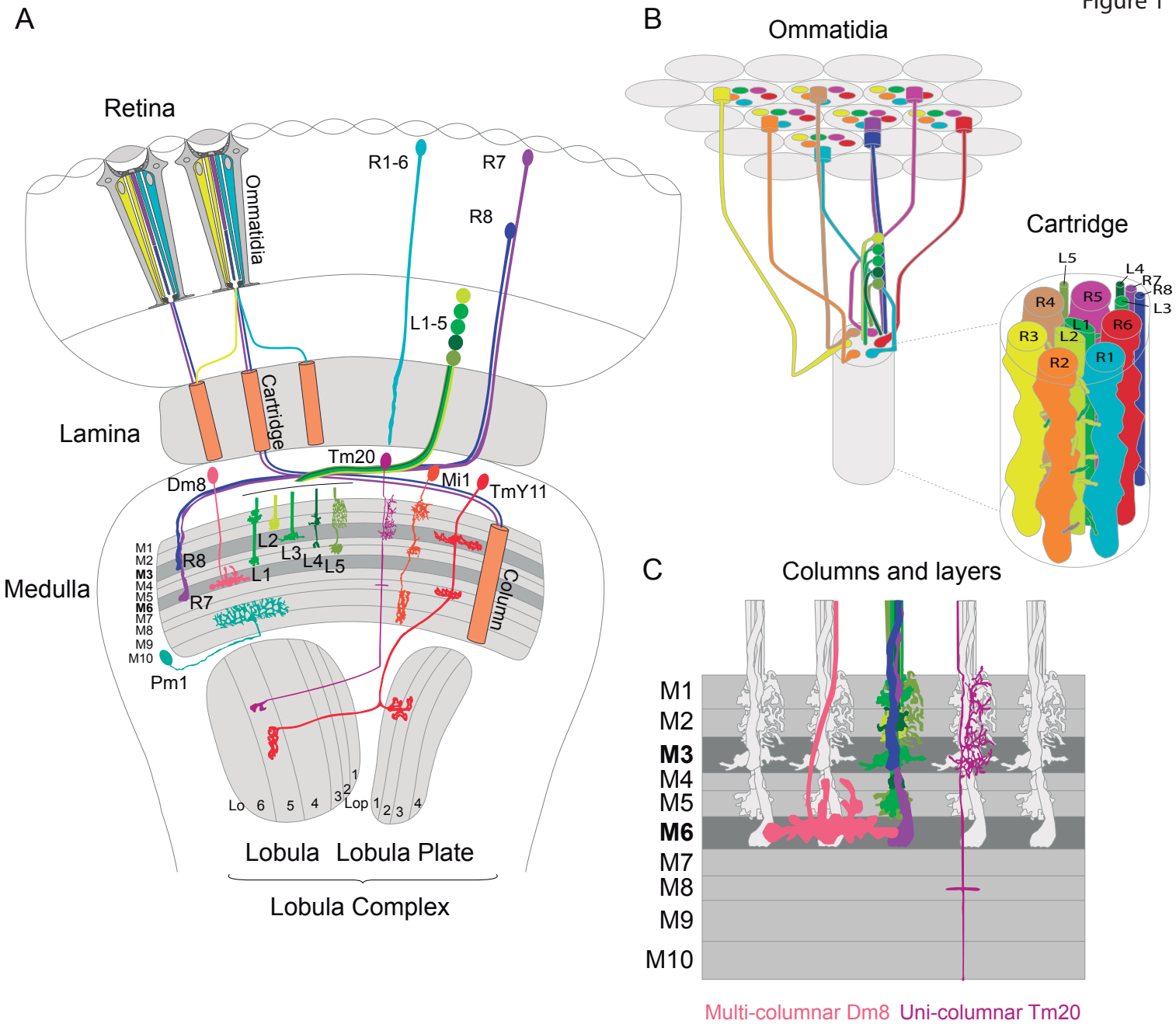
secreted from R8 axons acts in a paracrine anterograde fashion to regulate the dendritic field of the Tm20 medulla neuron, a major postsynaptic partner of R8. *babo* and *smad2*-mutant Tm20 dendrites extend into neighboring columns. **(C-D)** Cell–cell contact events between processes of neurons is another type of mechanism that regulates wiring. **(C)** Dscam2 homophilic interactions, which result in repulsive signaling, between neurites of L1 lamina neurons in neighboring columns during development are important to limit their arborization to their own column. The absence of Dscam in one cell causes a non-autonomous side-specific phenotype in the neighboring cell: wild-type dash-outlined L1 neuron dendrites invade the column where the mutant L1 neuron is present. **(D)** CadN homophilic adhesive interactions regulate L5 arbor extensions at M2 and M5. The fact that *CadN*-mutant L2 neurons (outlined in red) result in an L5 non-autonomous phenotype indicates that CadN interactions between these two neurons shape L5 dendritic arborization at M2. Other neurons with processes at M5 and expressing CadN are candidates to limit L5 arborizations at M5 to the home column.

**Figure 4. Formation of medulla layers.** Diagram representing the development of medulla layers along time and axonal behaviors of the R7 and R8 photoreceptors. Layers were initially defined by the branching of neurons at specific location along the Z-axis of the medulla (Fischbach & Dittrich, 1989). Layers can be recognized by labeling the neuropil with molecules enriched at synaptic sites and a combination of cell specific markers. The diagram presented here (adapted from Ngo, Andrade & Hartenstein, 2017) reconstructs the development of the medulla layers based on CadN expression (Ngo et al., 2017). The lighter the grey color, the higher NCad expression. Dark grey regions represent expression domains with the lowest CadN expression. At late 3$^{rd}$ instar larval stages the developing medulla represents a single protolayer with

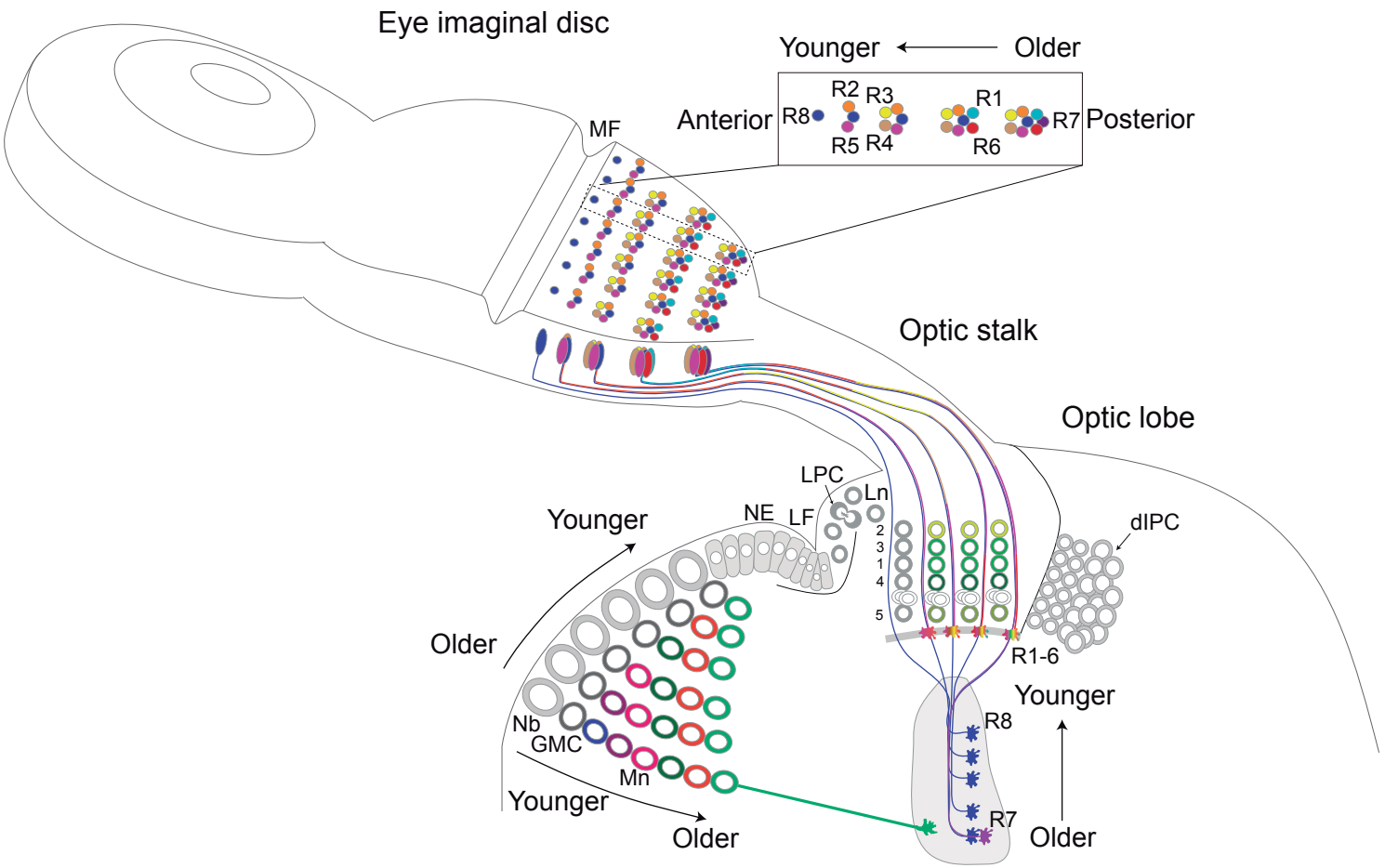
homogeneous CadN staining. At 24h APF the medulla is divided in two CadN staining regions separated by a band of low signal. The upper band corresponds to the protolayer for M1 to M6, the lower band to the protolayer M9-M10. This lower band does not experience major changes in CadN signal over time. At 48 h APF the protolayer for M1-M6 has further stratified with the appearance of a thin band of low CadN. The top band corresponds to the protolayer for M1 and M2. The lower band represents the primordium of the M4 to M6 layers. The low CadN band comprising the future M7-M8 layers gets separated by a band of very low CadN staining, which demarcates the M7/M8 boundary. At 72 h APF the pattern of CadN resembles the adult expression. Major changes compared with the earlier stage include: the subdivision of the M3 layer in a middle stratum with a thin band of CadN signal surrounded by two thin dark bands, and the very low expression of CadN in the M5 and M6 layers. The behavior of R7 and R8 growth cones along these time points has been studied both in fixed and in vivo samples. When R7 axons project to the medulla they do so to a region that seems to correspond to the same region they will occupy in the adult. Passive dislocation, axon elongation, and specific growth cone dynamics as the medulla grows due to innervation by other neurons explain R7 layer selection. The R8 axon initially projects to the edge of the medulla and actively extends from there to the M3 layer. The stereotyped behavior of R8 growth cones has been carefully described. After mid pupal development, when R7 and R8 have positioned themselves in the protolayers that will give rise to their adult layer termination pattern, both neurons will undergo further changes. In addition to axon elongation, their growth cones' transformation to synaptic terminals and synaptogenesis will proceed.

**Figure 5. Stepwise development and sequential axonal derived cues shape the wiring of neural circuits. (A)** Early on in the wiring of the fly visual system, afferent-derived cues shape the target region. The first example is the influence of photoreceptor signals in directly ensuring the formation of the right number of lamina neurons (through Hh); and, indirectly through wrapping glia (wg) (photoreceptor-derived EGF to glia, Ilp from glia to lamina neurons), their proper differentiation. Another instance is the effect of anterograde signaling on target survival as seen in panel B. **(B)** R cells mediate survival of L3 through Jeb/Alk signaling. R8 layer selection depends on L3 survival and the expression of Netrin, which will stabilize the R8 growth cone to its target layer. At the same time, the development of the medulla layers relies on cellular interactions between afferents. These interactions shape the medulla in a stepwise fashion as new afferents innervate the neuropil and protolayers evolve to the adult structure. In this way, L1, L3 and L5 project together, most probably through CadN homophilic interactions between them, to a common region delimited by Sema/PlexA-repulsive interaction between these lamina neurons and medulla transversal axons (MeT). Sema/Plex-repulsive interactions are also used to facilitate L3 sculpting and targeting of the proto M3. **(C)** All together, these interactions give rise to the adult layered medulla. The layered pattern is based on CadN expression, as in Fig. 4.

Figure 1


A
Retina
Ommatidia
R1-6
R7
R8
L1-5
Cartridge
Lamina
Tm20
Dm8
Mi1
TmY11
M1
M2
M3
M4
M5
M6
M7
M8
M9
M10
R8
L1
L2
L3
L4
L5
R7
Medulla
Column
Pm1
Lo 6 5 4 3 2 1 Lop 1 2 3 4
Lobula
Lobula Plate
Lobula Complex
B
Ommatidia
Cartridge
L5
L4
R7
R8
L3
R4
R5
L1
R6
R3
L2
R1
R2
C
Columns and layers
M1
M2
M3
M4
M5
M6
M7
M8
M9
M10
Multi-columnar Dm8
Uni-columnar Tm20

A


Eye imaginal disc
Younger
Older
Anterior
Posterior
R8
R2
R3
R5
R4
R1
R6
R7
MF
Optic stalk
Optic lobe
LPC
Ln
NE
LF
dIPC
Younger
Older
2
3
1
4
5
R1-6
Younger
R8
Nb
GMC
Mn
R7
Younger
Older
Older

B

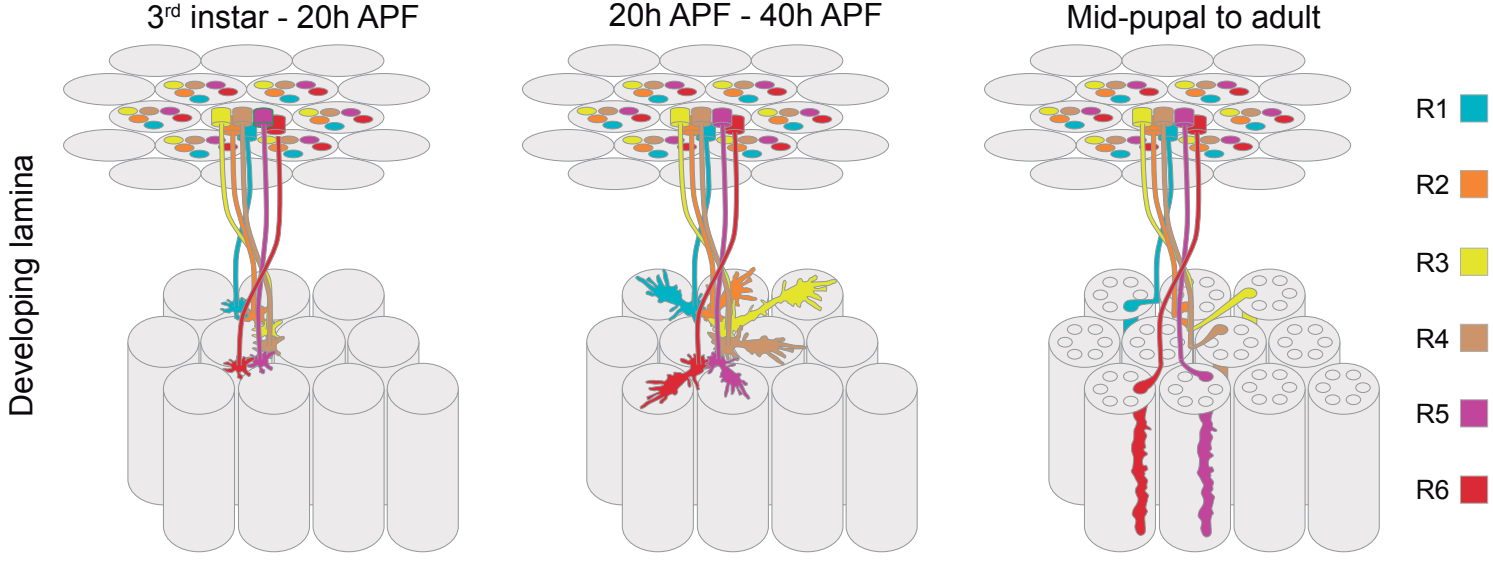
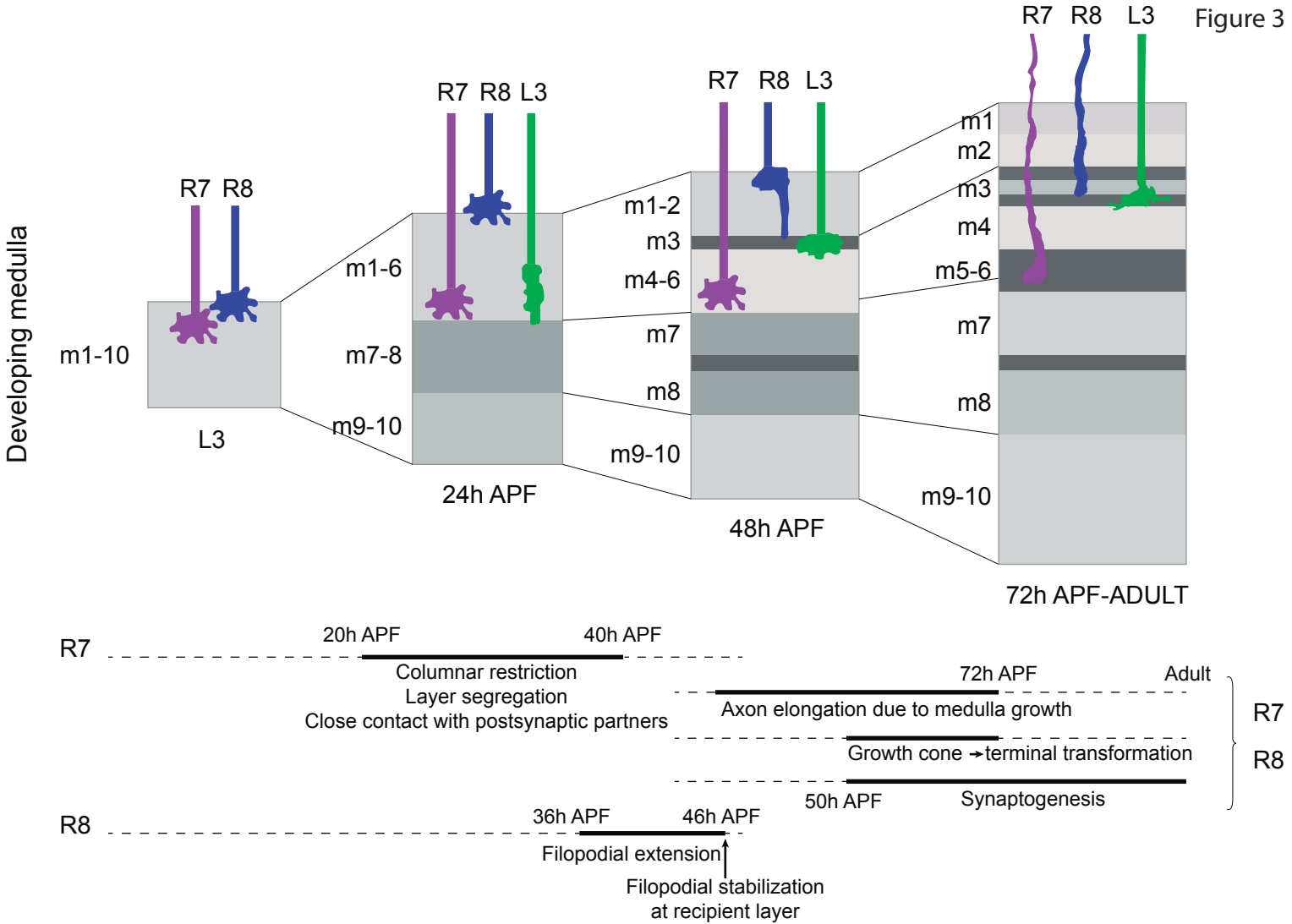
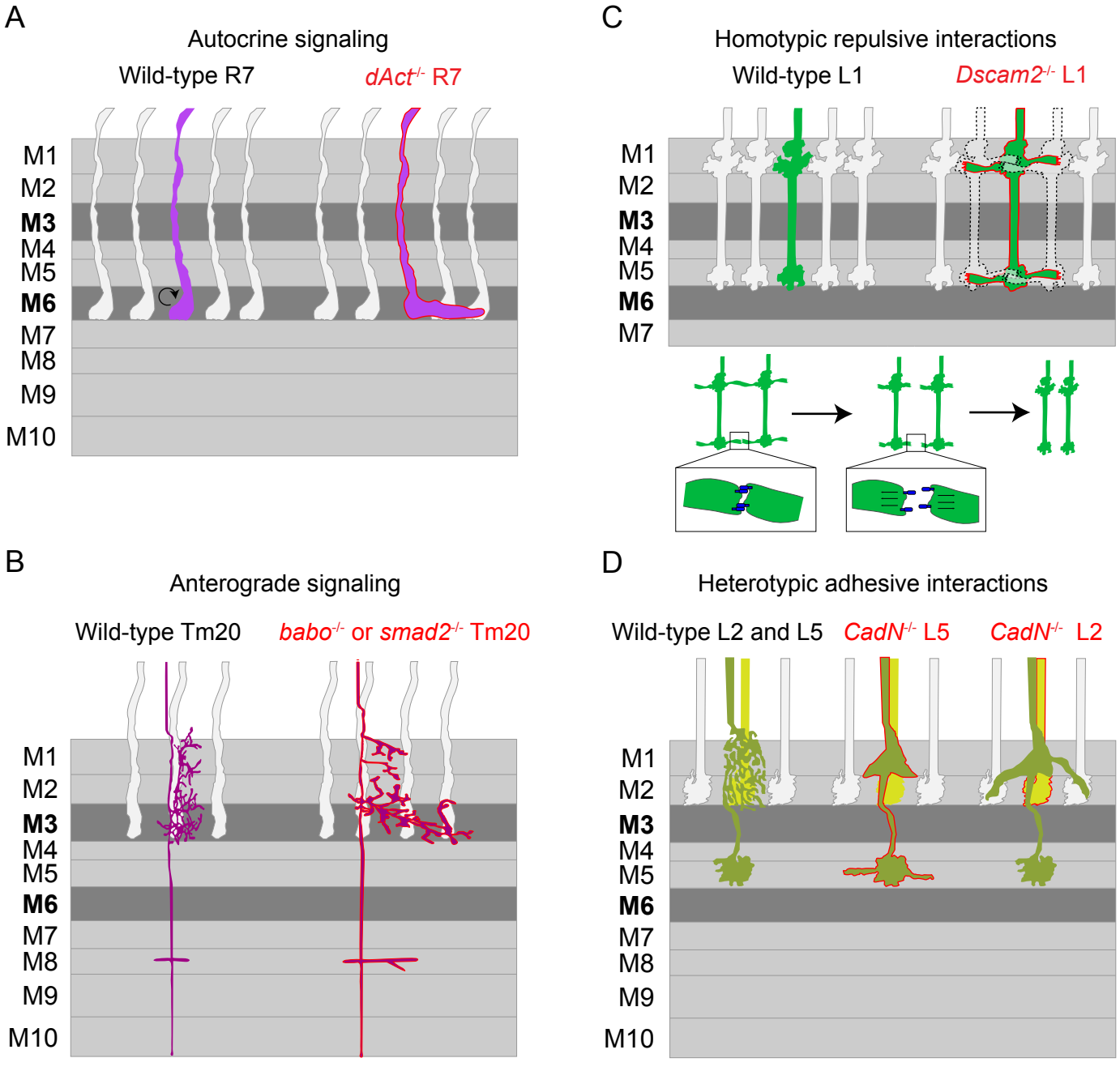
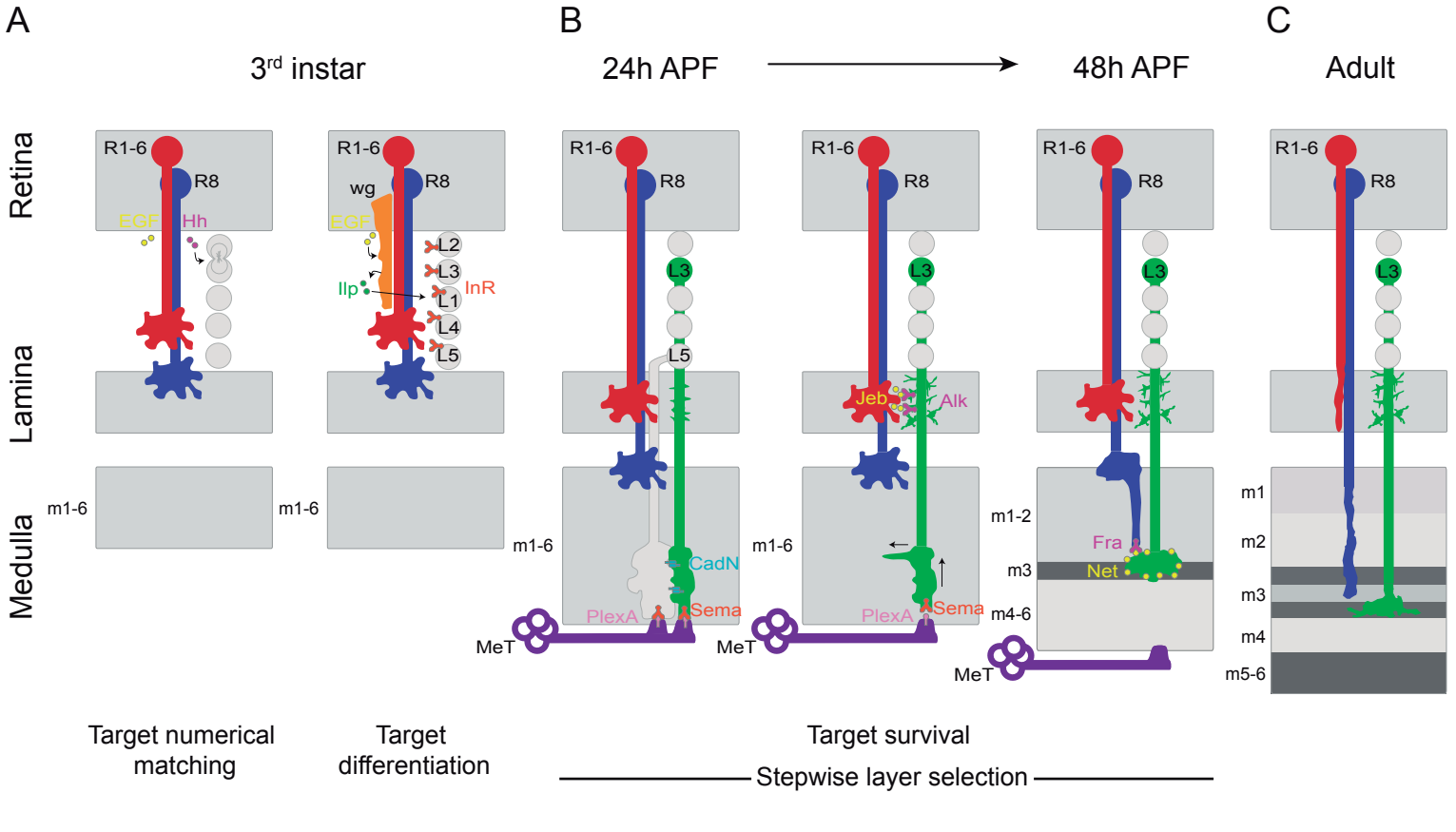

3rd instar - 20h APF
20h APF - 40h APF
Mid-pupal to adult
Developing lamina
R1
R2
R3
R4
R5
R6

Figure 3


Developing medulla
R7 R8
m1-10
L3
R7 R8 L3
m1-6
m7-8
m9-10
24h APF
R7 R8 L3
m1-2
m3
m4-6
m7
m8
m9-10
48h APF
R7 R8 L3
m1
m2
m3
m4
m5-6
m7
m8
m9-10
72h APF-ADULT
R7
20h APF
40h APF
Columnar restriction
Layer segregation
Close contact with postsynaptic partners
72h APF
Adult
Axon elongation due to medulla growth
Growth cone → terminal transformation
50h APF
Synaptogenesis
R7
R8
R8
36h APF
46h APF
Filopodial extension
Filopodial stabilization
at recipient layer


A
Autocrine signaling
Wild-type R7
dAct-/- R7
M1
M2
M3
M4
M5
M6
M7
M8
M9
M10
C
Homotypic repulsive interactions
Wild-type L1
Dscam2-/- L1
M1
M2
M3
M4
M5
M6
M7
B
Anterograde signaling
Wild-type Tm20
babo-/- or smad2-/- Tm20
M1
M2
M3
M4
M5
M6
M7
M8
M9
M10
D
Heterotypic adhesive interactions
Wild-type L2 and L5
CadN-/- L5
CadN-/- L2
M1
M2
M3
M4
M5
M6
M7
M8
M9
M10


A
B
C
3rd instar
24h APF
48h APF
Adult
Retina
Lamina
Medulla
R1-6
R8
EGF
Hh
wg
Ilp
InR
L1
L2
L3
L4
L5
m1-6
Jeb
Alk
CadN
PlexA
Sema
MeT
m1-2
m3
m4-6
Fra
Net
m1
m2
m4
m5-6
Target numerical matching
Target differentiation
Target survival
Stepwise layer selection