The Color-Vision Circuit in the Medulla of Drosophila

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Summary

Background: Color vision requires comparison between photoreceptors that are sensitive to different wavelengths of light. In Drosophila, this is achieved by the inner photoreceptors (R7 and R8) that contain different rhodopsins. Two types of comparisons can occur in fly color vision: between the R7 (UV sensitive) and R8 (blue- or green sensitive) photoreceptor cells within one ommatidium (unit eye) or between different ommatidia that contain spectrally distinct inner photoreceptors. Photoreceptors project to the optic lobes: R1–R6, which are involved in motion detection, project to the lamina, whereas R7 and R8 reach deeper in the medulla. This paper analyzes the neural network underlying color vision into the medulla.

Results: We reconstruct the neural network in the medulla, focusing on neurons likely to be involved in processing color vision. We identify the full complement of neurons in the medulla, including second-order neurons that contact both R7 and R8 from a single ommatidium, or contact R7 and/or R8 from different ommatidia. We also examine third-order neurons and local neurons that likely modulate information from second-order neurons. Finally, we present highly specific tools that will allow us to functionally manipulate the network and test both activity and behavior.

Conclusions: This precise characterization of the medulla circuitry will allow us to understand how color vision is processed in the optic lobe of Drosophila, providing a paradigm for more complex systems in vertebrates.

…to extend our understanding of neural function to the most complex human physiological and psychological activities, it is essential that we first generate a clear and accurate view of the structure of the relevant centers, and of the human brain itself, so that the basic plan—the overview—can be grasped in the blink of an eye.”

—Santiago Ramón y Cajal, circa 1904 [1]

Introduction

During evolution, eyes have been optimized to process maximum amounts of information by perceiving different parameters of the visual world and responding to them. The eyes can perform several major functions, from simple detection of light for circadian clock entrainment to formation of images. The eyes also can achieve other visual functions in which the quality of the light input, such as color, or the vector of polarized light (for navigation purposes) also is considered. The image of the environment formed by the optics of the eye on the retina is then transferred to brain processing centers. Both retina and brain processing centers have specialized morphology and function to achieve their various tasks (for a review, see [2]).

The Drosophila compound eye is composed of ~800 ommatidia, each containing eight photoreceptor cells named R1 to R8 (for a review, see [3]). In each ommatidium, the six “outer photoreceptors” (R1–R6) contain the wide-spectrum Rhodopsin Rh1, which allows them to absorb light efficiently for their specialized function in motion detection (for a review, see [4]). Tests based on associative-learning strategies have demonstrated color vision in Drosophila (most recently, [5]). The R7 and R8 “inner photoreceptors” function as sensory receptors for this color-vision system [6, 7]. They contain different types of Rhodopsin (Rh3–Rh6). Based on the Rh content of their inner photoreceptors, two main classes of ommatidia can be distinguished that are distributed stochastically in the main part of the retina [8, 9], resulting in the formation of a mosaic of photoreceptors with different wavelength absorption (for a review, see [10]). Ommatidia exhibit unique fluorescence of their inner photoreceptors that appear either yellow (y) (70% of ommatidia) or pale (p) (the remaining 30%). The p-ommatidia contain UV-Rh3 in R7 and blue-Rh5 in R8, whereas y-ommatidia contain UV-sensitive Rh4 in R7 and green-sensitive Rh6 in R8. These two subtypes of ommatidia presumably allow discrimination of a broad range of wavelengths, with the p-ommatidia involved in the discrimination among short wavelengths, whereas y-ommatidia must be involved in discriminating among longer wavelengths.

The adult Drosophila optic lobe has approximately 60,000 cells and can be divided in four neuropil layers (for review [11]): lamina, medulla, lobula and lobula plate (Figure 1A). The medulla represents the largest structure in the optic lobe with an estimated 40,000 neurons [12] whose cell bodies are located either in the medulla cortex, the region between the lamina and the medulla neuropil (arrows in Figure 1A), or the medulla rim, the region between the medulla and the lobula plate (arrowheads in Figure 1A). The medulla neuropil is stratified in 10 layers (M1–M10) orthogonal to the orientation of photoreceptor projections [13]. They can be divided in two larger domains: M1–M6 are where R7 and R8 send their projections (Figure 1B) while M7–M10 are lower medulla layers that are devoid of photoreceptors projections (brackets in Figure 1B).

Thus, the medulla is the first step in the color-vision pathway because it receives direct input from R8 to the M3 layer and R7 to the M6 layer [11]. It is, however, the second step in the motion-detection pathway from R1–R6 via input of lamina monopolar neurons to the medulla [14]. Therefore, the medulla likely couples the motion-detection and color-vision pathways. Corresponding to each set of R7/R8 projections, there are ~800 “columns,” defined as fixed cassettes of cells that repetitively contact each R7–R8 fascicle. Columns likely represent the functional units in the medulla with “columnar neurons” contacting photoreceptors from only one ommatidium. “Noncolumnar neurons” presumably integrate information...
Figure 1. Transcription Factor Expression Patterns in the Medulla

(A) Adult optic lobe showing neurons (Elav, blue) in the medulla (Me) cortex (arrows) and medulla rim (arrowheads), lobula (Lo), and lobula plate (LP). (B) High magnification of medulla layers. Brackets show lower medulla layers (M7–M10). (C) Expression pattern of ey-Gal4 driving UAS-CD8::GFP (green). Brackets show lower medulla layers and arrows axonal projections to the lobula. (D) Expression pattern of ap-Gal4. Arrowheads point to L4 neurons and arrows to axonal projections to the lobula. Brackets show medulla layers. (E) Expression pattern of dll-Gal4. (F) Expression pattern of c699-Gal4. Brackets in (E) and (F) show ramifications in the lamina. (G) Nonoverlapping expression of ey-Gal4 driving UAS-nuGFP (green), Distal-less antibody (red), and ap-lacZ (blue) in medulla neurons. Inset shows high magnification of medulla cortex. (H) Expression of ey-dll/c699-Gal4 driving UAS-nuGFP (green) and ap-lacZ (red) in medulla neurons (blue). Photoreceptor projections visualized with glass-lacZ (green) in (A) and (B), monoclonal antibody 24B10 (blue) in (C)–(F), and neuropil stained with DN-Cadherin (red) in (A)–(F). Scale bar: 50 μm in (A) and (C)–(H) or 15 μm in (B).
coming from broader receptor fields. Fischbach, Cajal, Strausfeld and colleagues extensively described and categorized medulla cells in *Drosophila*, *Calliphora*, and *Musca*, respectively [13, 15, 16]. In these investigations, Golgi impregnation was used to reconstruct the morphology of optic lobe cells. Although immensely useful, this technique has limitations due its inability to identify landmarks (e.g., photoreceptors). This makes it difficult to correlate the position and contacts of different cells with the photoreceptors that provide the input to the system or among medulla neurons, and to define their polarity.

By using the mosaic analysis with a repressible cell marker (MARCM) technique [17] and a series of enhancer traps Gal4 lines reporting the expression of several transcription factors (TFs) and by generating a collection of piggyBac Gal4 enhancer trap lines specific for many of the individual cell types, we have been able to reconstruct the morphology of most individual medulla neurons (Table 1). Based on morphology, probable connection patterns and neuronal polarity, we focus on five broad classes of neurons: columnar and noncolumnar projection neurons that reach the lobula and the lobula plate, local columnar and noncolumnar local neurons with ramifications limited to the medulla, and feedback neurons that project back to the lamina. Although many of these neuronal classes have previously been identified [13], our precise characterization and the identification of new classes allow us to construct a possible neural circuitry that could be the basis for color vision in *Drosophila*.

### Results

#### Transcription Factors Expressed in Medulla Neurons

In order to obtain a complete description of medulla neurons, we identified broad molecular markers for all neurons in this structure. Extensive work on the development of the mammalian retina has led to the identification of a network of TFs expressed and required in specific retinal cell types [18]. The fly and vertebrate eyes use similar developmental pathways that were presumably involved in the formation of photoreceptors in their common ancestor [19]. Therefore, we looked at the expression pattern in the medulla of the fly orthologs of these

### Table 1. Medulla Cell Types

| Neuronal cell types identified in the Drosophila medulla. Cell types are organized according to (1) their expression of a TF-Gal4 (ap-, ey- and dll-Gal4 lines), (2) columnar (C) versus noncolumnar (NC) cells, and (3) the presence of one dendritic arbor contacting R7 or R8, two arbors (R7 and R8), or ramifications in the lower medulla layers (LM). Numbers in parentheses represent the number of cells examined for each cell type. Abbreviations: P&Y, pale and yellow ommatidia; OT, optic tubercle; and Lo, lobula. New cell types are shown in bold. Cells for which we describe a specific Gal4 enhancer trap line are noted. |

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TFs by using enhancer trap Gal4 driver lines crossed with a membrane-bound UAS-CD8::GFP reporter (Figures 1C–1E).

An enhancer trap in the eyeless gene (eyGal4) [20], the Pax-6 “eye-master-control gene” [21], drives reporter gene expression in ~30% of medulla cortex neurons (Figure 1C) (Table 1). Two enhancer trap lines in aperturoid (apGal4 [22] and ap-lacZ [23]), which encodes a LIM-homeoprotein homologous to mammalian Lhx2, are expressed in L4 lamina cells (arrowheads in Figure 1D) and in ~40% of cells in the medulla cortex (Figure 1D) (Table 1). Both ey-Gal4 and ap-Gal4 are expressed in medulla “projection neurons” that reach to deeper optic lobe neuropils (arrows in Figures 1C and 1D), but they appear to mark different subsets of neurons whose ramifications occupy distinct domains in the medulla: ey-Gal4 preferentially marks cells that contact lower medulla layers (brackets in Figure 1C), whereas processes of ap-Gal4 cells are present throughout the entire medulla (brackets in Figure 1D). An enhancer trap in distal-less, dlmmd23-Gal4 [22], shows expression in ~20% of medulla cortex cells and in medulla rim cells (Figure 1E) (Table 1). These cells appear to be medulla local neurons, whereas a subpopulation sends ramifications back to the lamina (brackets in Figure 1E). Finally, c699-Gal4 [24] is expressed in only one medulla cell type, identified as T1 cells [13], representing ~5% of medulla cells (Figure 1F). This cell type does not express any of the other three transcription factors (Table 1).

As these Gal4 lines are expressed in several subtypes of medulla neurons, we analyzed whether they are coexpressed in the same cells. None of these TF markers overlap (ey-GFP, ap-lacZ and anti-Dll [25]), both during larval development (our unpublished data) and adulthood (Figure 1G and inset). Furthermore, flies carrying a combination of three Gal4 lines (c699-, dlm- and ey-Gal4) driving nuclear GFP as well as ap-lacZ mark over 95% of medulla neurons (Figure 1H). The remaining classes seem to be labeled by 192a-, 782c-, 2402a-Lex-Gal4 and 1118-Gal4 lines expressed in very restricted groups of medulla cells (see below and “other cell types” in Table 1). These markers not only are expressed in mutually exclusive subsets of neurons but also, more importantly, they mark the large majority of medulla neurons.

Visualizing Neuronal Subtypes in the Medulla

Visual inputs flow from the retina to the lamina or to the medulla before reaching downstream targets in the lobula complex. Therefore, the medulla presumably represents an intermediate structure in which projection neurons carry visual information toward deeper regions of the optic lobe.

Precise anatomical studies using Golgi impregnation in Drosophila have identified two broad classes of neurons in the medulla [13]. (1) Projection neurons connecting the medulla with either only the lobula (transmedullary neurons, Tm) or with both lobula and lobula plate (TmY neurons, which have branched axons) or projecting back to the lamina. These projection neurons constitute the main output pathway from the medulla and are considered the equivalent of the mammalian retinal ganglion cells [15]. (2) Local interneurons with ramifications limited to the medulla. In order to reconstruct the neural network in the medulla, we generated MARCM GFP-clones [17] of projection and local neurons by using the Gal4 lines described above (Figure 1) and UAS-CD8::GFP that decorates both axonal and dendritic ramifications. Examination through confocal microscopy of more than ~10,000 adult brains has allowed us to reconstruct the morphology and pattern of projections of 2000 single-cell clones in frontal sections, re-identifying those neurons previously described [13] and expanding to a large set of new neurons. In order to have precise morphological landmarks, we labeled all R7 and R8 projections with a monoclonal antibody against Chaoptin (24B10) [26] and the optic lobe neuropil with DN-Cadherin [27]. We were then able to reconstruct cell ramifications and precise morphology for each neuron type present in the medulla (Table 1).

We describe below a selected number of representative examples of the types of neurons that we identified. We used the nomenclature from Fischbach and Dittrich [13] for known cell types and we adjust it to name newly identified cells by considering the medulla layers where major ramifications are displayed (Table 1).

Vertical Integration: R7 and R8 Integration

In mammals, color vision requires the comparison between cones expressing different opsins in order to distinguish between wavelength and intensity contrasts (for a review, see [28]). The organization of the fly retina, with UV-sensitive R7 located on top of green- or blue-sensitive R8, allows both inner photoreceptors within one ommatidium to look at the same point in space. We find a broad class of columnar Tm cells (Table 1) with ramifications in both R7 and R8 layers (Figures 2A and 2B), which might be able to compare the output of R7 and R8 photoreceptors, such as in a color opponent system (vertical integration). The previously unidentified Tm cells that we name Tm2a6 (Figure 2A) are representative of this class. They have ramifications in the M3 and M6 layers (arrowheads in Figure 2B) and also present ramifications in lower medulla layer domains (M7–M10) (arrows in Figure 2A), which appear to be common features of almost all medulla neurons.

We found a second subclass of columnar neurons that present ramifications in a single photoreceptor terminal layer (Figures 2C and 2D) (Table 1). For instance, Tm2 cells (Figure 2C) exhibit extensive contacts in the M2–M4 layers (Figure 2D), with two lateral ramifications surrounding R8 terminations (arrowheads in Figure 2D). They bypass R7 termini (M6 layer) (arrow in Figure 2D) when projecting deeper toward the lobula. They also send a second set of small ramifications in the M9 lower medulla layer (arrow in Figure 2C). Tm2 cells express ort (C.-H. Lee, personal communication), the gene that encodes the receptor for histamine (the neurotransmitter of photoreceptors), and, therefore, presumably receive inputs from either R8 or R7. The ramifications displayed by this subclass in M1–M5 [13] correspond to layers where lamina neurons also project, arguing that these neurons might be involved in integration with motion detection before the information is sent to downstream lobula targets.

Horizontal Integration: p- and y-Ommatidia

The single lens, camera-like mammalian eye forms images on the retina, where rods and cones are displayed in a two-dimensional continuous layer: comparison for color vision occurs between neighboring cones. Instead, in Drosophila, each pixel corresponds to an ommatidium that has a dedicated lens, and the color of the pixel could be inferred from comparisons in columnar neurons that contact R7 and R8 from one ommatidium. However, we also find a family of noncolumnar projection neurons (with more than ~20 types) that project to the termini of R7/R8 belonging to several neighboring ommatidia (as in vertebrates). These neurons present arborizations with different complexity in several photoreceptor layers and in their axonal projections to the lobula complex. They might perform
"horizontal" comparison between pale and yellow ommatidia (Figures 2E–2J).

The first subclass includes cells that contact both R7 and R8 in several columns. These cells present at least 11 different variations based on dendritic arbor morphology in photoreceptor layers (Table 1). They penetrate the medulla along a specific R7/R8 column and then expand ramifications in photoreceptor layers covering from two to ten columns, both in the R8 and R7 layers. Like other projection neurons, they also contact lower medulla layers M7–M9 (arrow in Figure 2F). Figures 2E and 2F show one such neuron that resembles Tm5 [13]. Different subclasses differ whether their axons project only to the lobula (e.g., Tm5) (Figure 2E) or also to the lobula plate (Table 1).

The second subclass with at least nine different variations contains neurons with only one tuft of lateral ramifications that contact either several R7 or several R8 photoreceptors (Figures 2G–2J). For instance, TmY2 [13] only arborizes in the R8 layer (Figures 2G and 2H). A previously unidentified cell type, which we name TmP&Y R7, arborizes only in the R7 layer (Figures 2I and 2J). Both subtypes have ramifications in M7/M8 lower medulla layers (arrows in Figures 2H and 2J).

Local-Circuit Neurons
Visual processing must involve interneurons, or "local-circuit neurons," which might shape the outputs generated by projection neurons. We identify two broad classes of columnar and noncolumnar local neurons, which extend distinct ramifications exclusively in the medulla (Table 1).

The first class of local neurons are columnar Medulla intrinsic (Mi) cells [13] (Table 1). Like columnar projection neurons, these local neurons have branches at two different levels in the photoreceptor layers (Figures 3A and 3B) or exhibit arborizations branching out in one main tuft (Figure 4C). It is noteworthy that the ramifications of local neurons often resemble and coincide with those of corresponding projection neurons, arguing for functional relationship between them. For instance, columnar local Mi1 and projection TmY5a neurons have similar ramifications in M1, M3 (R8), and M5 layers (Figures 3A–3D).

We also find two subclasses of noncolumnar local neurons. Distal medulla (Dm) neurons [13] have ramifications in photoreceptor layers, whereas Proximal medulla neurons (Pm, described below) [13] extend arbor in lower medulla domain. We find Dm cells whose morphology closely matches that of almost every class of noncolumnar projection neurons described above (Table 1). For instance, a previously unidentified noncolumnar local neuron that we name Dm1–5 and projection neuron TmY8 share ramifications in M1, M4 and M5 layers (Figures 3E–3H). Their only difference is that TmY8 projects to the lobula complex, whereas Dm1–5 remains local.

Axons in Projection and Local Neurons
The presence of matching pairs of local and projection neurons suggested a functional relationship between them. We thus analyzed the pre- and postsynaptic nature of terminations in those classes of neurons. First, we used single-cell
MARCM clones to coexpress UAS-CD8::GFP along with a reporter of a different color that marks axons (Tau::lacZ) (Figure 4).

In columnar Tm cells, Tau::lacZ accumulates in lobula and lobula plate projections (e.g., TmY5a, arrowheads in Figure 4A) and faintly in lower medulla layers, but not in lateral ramifications in photoreceptor layers, which are thus likely dendrites (arrows in Figure 4B). In contrast, MARCM analysis of local neurons such as Mi9 shows Tau::lacZ clearly enriched in lower medulla arborizations and in photoreceptor layers, although some fine ramifications are also tau-negative in photoreceptor layers (Figure 4C), arguing for the presence of both pre- and postsynaptic contacts in photoreceptor layers.

We then analyzed the pre- and postsynaptic nature of terminations in noncolumnar projection and local neurons. Similar to columnar projections neurons, noncolumnar cells such as TmY7 accumulate Tau::lacZ in the main axis (Figure 4D and inset), but not in lateral ramifications in photoreceptor layers (Figure 4E). In contrast, equivalent noncolumnar local neurons such as Dm1–5 do not show a clear separation of axon and dendrites as shown by extensive enrichment of Tau::lacZ in their medulla ramifications, especially in the M1 layer (Figure 4F).

Therefore, Tm projections neurons appear to collect visual information from photoreceptors and send it to the lobula complex. Local neurons are both pre- and postsynaptic in photoreceptor and lower medulla layers where they likely modulate the information carried by projection Tm cells.

**Pre- and Postsynaptic Sites in Medulla Cells**

To confirm results described above, we used specific lines to mark unique cell populations in the medulla. We screened available Gal4 lines and a collection of piggyBac Gal4 lines generated in our lab and in the lab of T. Tabata (Figures S1 and S3). Available lines are indicated in Table 1 and will be described in detail elsewhere (A. Celik, J.M, T. Tabata, and C.D., unpublished data). We coexpressed UAS-CD8::GFP along with a reporter of a different color that marks presynaptic sites (Synaptotagmin::HA) (Figure 5). We also used UAS-DsRed together with a dendritic compartment marker (Dscam 17.1::GFP) (Figure 5).

In noncolumnar Tm12 cells (Table 1), marked with the 2135a-Gal4 line (Figures S1A and S1B), Dscam 17.1 is expressed in ramifications in photoreceptor layers (arrows in Figure 5B) and lower medulla layers (arrows in Figure 5A), but it is absent from axonal projections to the lobula (arrows in Figure 5A). This confirms that these are dendrites and this pattern is largely complementary to that of Tau::lacZ. Presynaptic sites labeled with Synaptotagmin::HA (Syt) are localized in lobula projections (arrows in Figure 5C). Interestingly, they are also found in a few ramifications in lower medulla layers, although most ramifications there are marked with Dscam 17.1::GFP (arrows in Figure 5B).

For local neurons, we used the specific 782c-Gal4 line (Figures S1C and S1D) that drives expression in noncolumnar Dm6 cells. These cells exhibit arborizations in M1 layer. Specific expression of Dscam 17.1 (Figure 5E) and Syt (Figure 5G) reveals...
enrichment of presynaptic sites (brackets in Figure 5H) in the arborizations that contact the M1 layer.

Third-Order Neurons: Role of Lower Medulla Layers

Projection neurons described above, whether columnar or not, appear to contact photoreceptors and likely represent a first level of color integration. All of these Tm neurons also have presynaptic sites (Figure 5) in lower medulla layers (M7–M10) beyond photoreceptor layers, where a higher level of integration might take place. Interestingly, although we do not find columnar projection neurons that bypass photoreceptor layers, we find two subclasses of noncolumnar projection neurons that do not contact photoreceptors and appear to act as third-order neurons in the medulla network.

A class of previously unidentified Tm cells, which we name TmLM7 (Figures 6A and 6B), arborize extensively in the M7 layer (Figure 6B), where they appear to be postsynaptic (see below) before projecting to the lobula. A second class includes T3 cells, whose cell bodies are in the medulla rim [13] (Figure 6E). They have axons that split in two branches, one reaching the deepest medulla layers (M9–M10) (arrow in Figure 6F), covering between two and four columns, whereas the axonal branch reaches the lobula.

We also find several types of noncolumnar local neurons (Table 1), known as Proximal medulla (Pm) neurons [13], which skip photoreceptor layers and only contact lower medulla layers. PmLM7 exhibit ramifications mainly in the M7 layer (Figures 6C and 6D), whereas PmLM9–10, whose cell bodies are in the medulla cortex (Figures 6G and 6H), project to the M9 and M10 layers. Interestingly, PmLM7 and TmLM7 cells have similar ramifications in the same layers (compare Figures 6A and 6B with Figures 6C and 6D), whereas PmLM9–10 neurons have projections that are similar to T3 neurons (compare Figures 6E and 6F with Figures 6G and 6H), further emphasizing the parallel between projection and local neurons.

We then analyzed the pre- and postsynaptic nature of terminations in these third-order neurons. In MARCM single-cell clones marking TmLM7, Tau::lacZ is enriched in lobula projections and in some, but not all, projections in the lower medulla domain, with the finest neurites being tau-negative, thus, presumably dendrites (Figures 6I and 6J). Similarly, Tau::lacZ is clearly enriched in the lower medulla arborizations of PmLM9–10 cells (Figures 6K and 6L), with some fine ramifications being negative. We expressed Dscam 17.1 and Syt using a Pm1-cell-specific line, 2402a-Gal4 (Figures S1E and S1F) (Pm1 differ from PmLM7 because the cell bodies are in the medulla rim).
Both dendrites (Figures 5I and 5J) and presynaptic sites (Figures 5K and 5L) can be observed in lower medulla layers, although presynaptic sites appear more abundant (brackets in Figure 5L).

Local neurons that do not contact photoreceptor layers likely perform further processing, which is then contributed to higher brain centers through second or third-order projection neurons. The postsynaptic nature of some of the lower medulla ramifications of third-order neurons suggests that these cells collect visual information already processed by other medulla cells with input from photoreceptor layers (either Tm or local neurons) and, thus, represent a higher level of hierarchy in visual processing.

**Projections to Lobula**

In the human retina, retinal ganglion cells carry visual information to higher optic centers. Similarly, projection neurons send their axons to higher visual processing centers in the lobula complex. We analyzed the axonal projection patterns in the lobula of individual medulla Tm neurons. Although lobula layers are morphologically less well defined than in the medulla, it is possible to define regions where medulla neurons project (Figure 7).

Columnar Tm cells that contact only one photoreceptor layer (TmR7/8) (e.g., Tm1, Tm2, and Tm9) show very discrete axonal projections with small termini in the most superficial lobula layers (Figures 7A and B). We confirmed this result by looking at all axonal projections of Tm2 cells which are marked by an ot-gl4 promoter fusion (T. Cook and C.D., unpublished data) (brackets in Figure 7B). However, columnar neurons contacting several photoreceptor layers (e.g., Tm3 cells, which contacts both M1 and M4 layers) project deeper, to an intermediate layer (Figure 7C).
Noncolumnar Tm cells, whether or not they contact photoreceptor layers (Figure 2) also present more complex axonal termini in the lobula, with multiple branches in layers deeper than columnar neurons (Figures 7D–7F). The depth of their projections in the lobula also directly correlates with the depth of their projection in the medulla. For instance, noncolumnar Tm cells that contact several photoreceptors (e.g., Tm5) (Figure 7D) or Tm12 cells marked with the 2135a-Gal4 line (brackets in Figure 7E) project more superficially than those that do not contact photoreceptors but have ramifications in the lower medulla (e.g., TmLM8), which project to the deepest layer in the lobula (Figure 7F).

Thus, lobula layers exhibit a hierarchy in the level of processing, with more superficial layers receiving signals with presumably lower level of processing whereas deeper layers are innervated by third-order medulla neurons and noncolumnar neurons. These data emphasize the spatial segregation of the different subtypes of projecting neurons to different lobula targets: visual information carried by columnar and noncolumnar cells is processed in separate lobula layers, which might represent the hierarchy of processing.

### References
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Neurons Connecting Medulla and Lamina

The projection neurons described above are not the only output of medulla neurons. Indeed, other cells such as T1, C3, and Lamina wide field (Lawf) neurons connect medulla and lamina [13]. These cell types show a high degree of conservation among insects [32], where they have been suggested to be part of a feedback circuit [13, 33].

By using the dll-, c699- and 1118-Gal4 [34] enhancer trap lines, we generated single MARCM single-cell clones of C3 (Figures S2G–S2I) neurons, respectively. To analyze their polarity, we expressed Dscam 17.1 and Synaptotagmin with the 1675a-Gal4 (Figures S3 C and S3D), which specifically marks Lawf neurons. C3 show dendritic ramifications in M1 and M5 layers (brackets in S4B), and Lawf neurons mainly in the M4 layer and faintly in the M1 layer (brackets in Figure S4F). However, Dscam 17.1 does not show staining in the lamina in either cell types (arrows in Figures S4B and S4F). C3 cells and Lawf cells show presynaptic sites (as shown with Syt-HA staining) in the lamina (arrows in Figures S4D and S4H). However, C3 cells show also some presynaptic sites in M1–M2 layers (brackets in Figure S4D) whereas Lawf cells have significant presynaptic termini in M1 and some in M4 (brackets in Figure S4H). Thus, the flux of information in these connecting neurons confirms previous reports that C3 and Lawf cells are part of a feedback pathway from the medulla to the lamina [33]. The role of T1 cells remains unknown. They do not appears to have presynaptic sites in the lamina and likely serve as a relay for L2 lamina neurons [14].

### Neurons Connecting Medulla and Lamina

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Figure 7. Axonal Projections to the Lobula
Axonal innervation (green) of Tm2 (A), Tm3 (C), Tm5 (D), and TmLM8 (F) MARCM single-cell clones to the lobula (between dashed lines). Expression pattern of otd-Gal4 (B) driving UAS-CD8::GFP (green) in Tm2 cells and photoreceptors or 2135a-Gal4 in Tm12 cells (E). Brackets in (B) and (E) point to axonal projections in the lobula. Photoreceptors are visualized with 24B10 (blue) and neuropil with DN-Cadherin (red). Abbreviations: Lo, lobula; LM, lower medulla; LP, lobula plate; P, pale; and Y, yellow. Scale bar: 50 μm.
Discussion

Analysis of the Visual System in Drosophila

The medulla represents the major neuropil in the optic lobe. Despite this complexity, by using a series of TFs-Gal4 lines we have been able to dissect the medulla network at the single-cell level. ap- and ey-Gal4 are expressed in nonoverlapping populations, both in projection and local neurons. dll-Gal4 reveals expression almost exclusively in local neurons, although not all local neurons are marked by dll-Gal4 (Table 1). Through this extensive analysis we have been able to reconstruct morphologically 38 types of projection neurons, 22 types of local neurons and 3 connecting neurons (Table 1).

Among these cell types, we found six new projection and four local neuron types not described before [13]. Most of these new cell types (e.g., TmLm7 and PmLm7) include cells with ramifications exclusively in the lower medulla domain.

We can thus define the elements represented in a “column,” the medulla functional units: (1) two inputs from R7 and R8, (2) five ramifications from L1–L5 lamina neurons, (3) 11 types of columnar and 20 of noncolumnar projection neurons, (4) four types of columnar and 11 of noncolumnar local neurons, and (5) two types of columnar and one of noncolumnar lamina connecting neurons. Additionally, seven other noncolumnar local and seven projections neurons do not contact PRs. Overall, each R7/R8 termination pair is therefore surrounded by at least 54 different cell types, with 14 other cell types that do not contact PRs. The enormous number of cell types forming part of a column contrasts with the relatively small number of elements that feed into the medulla (R7–R8 and R1–R6 through L1–L5 neurons). Thus, the divergence in the flow of information between PRs and medulla neurons argues that much local processing occurs in the medulla. In contrast, in the deeper optic lobe (i.e., lobula and lobula plate), the number of cells and their wide-field ramifications [13, 35] argue for the convergence of information from medulla neurons.

Vertical versus Horizontal Integration

Our analysis of the color-vision network in the Drosophila medulla reveals the presence of two parallel routes carrying and processing visual information that coexist in the medulla neuropil: (1) a point-to-point pathway formed by columnar neurons that only receive information from a single ommatidium (“vertical integration”) (Figure S5A), and (2) a pathway with a broader receptive field composed by noncolumnar neurons that receive information from photoreceptors from several ommatidia (“horizontal integration”) (Figure S5A).

The first pathway integrates outputs from R8 and R7, likely allowing broad wavelength discrimination between UV- and blue or green: cells comparing signals from p-ommatidia might mediate better discrimination among short wavelengths, whereas those comparing outputs of y-photoreceptors should better mediate discrimination among longer wavelengths. This suggests that there are two independent and nonoverlapping retinotopic maps that separately process color information: one from p- and one from y-photoreceptors. These maps might be physically separated in higher brain centers, but we do not observe specific p- or y-contacting neurons (our unpublished data), nor do we see distinct projections to different layers in the lobula complex (Figure 7B).

The second pathway reflects more complex visual processing, with noncolumnar Tm neurons cells integrating information from several R8 and R7 photoreceptors. Because the p- and y-ommatidia each compare output of photoreceptors with widely different absorption spectra (UV and green, or UV and blue), “horizontal integration” between y- and p-ommatidia for both R7 and R8 might allow a much more precise evaluation of the colored world, although at a reduced spatial resolution. Additionally, we find cells that might directly compare p- and y-R8 cells (e.g., contacting blue and green R8, but not R7). Similarly, cells that compare p- and y-R7 photoreceptors might integrate information from the two different types of UV-photoreceptors. It should be noted that the difference in peak of absorption between Rh3 and Rh4 (~20 nm) in R7 is sufficient to allow precise discrimination of UV wavelengths and is similar to the difference between M- and L-opsins in humans (30 nm). Thus, this horizontal integration should allow the convergence of several inputs from multiple R7 and R8 photoreceptors to a single medulla cell.

Modulation by Local Neurons

It is not clear how comparison between photoreceptors is achieved and whether local neurons expressing different neurotransmitters are involved to generate opposite outputs between R7 and R8 (for columnar neurons) or between p- and y-R7 or R8 (for noncolumnar neurons). It is likely that R7 cells do sum up their output to support the strong UV attraction that characterizes flies [36]. However, inner photoreceptors are not involved in scotopic vision (dim light), and an organization in which neurons simply add their outputs makes little sense for what is known of the function and specialization of R7 and R8 cells in color vision. Therefore, it is likely that an opponent system exists in the medulla and that it is mediated by local neurons. Alternatively, color vision might not need an opponent system but might result from nonlinear interactions between R7 and R8. Their interaction with the same postsynaptic cell could be complex, e.g., Tm neurons might have synergistic responses to inputs from R7 and R8 or from photoreceptors from different ommatidia.

In the mammalian retina, horizontal and amacrine cells are interneurons that have a critical role in modulating retinal output [2]. Horizontal cells provide lateral interactions between photoreceptor terminals, creating a “center-surround organization,” enhancing the response of ganglion cells lying directly under the light stimulus and inhibiting their neighbors. Meanwhile, amacrine cells not only make inhibitory synapses on bipolar cells, thus controlling their output to ganglion cells, but also synapse onto ganglion cells and coordinate their firing. As in the mammalian retina, a great variety of neurons with local ramifications within the medulla might modulate the visual outputs carried by projection neurons. This modulation is accomplished by two kinds of local neurons. (1) Columnar local neurons with one Mi cell per ommatidium (Figure S5B). Interestingly, the arborizations of many of these columnar local neurons resemble those of corresponding Tm columnar projection neurons that contact either one photoreceptor, or both R7 and R8. They intermingle with them in photoreceptor layers as well as in lower medulla layers. In this microcircuit, Mi cells appear to interact both pre- and postsynaptically with Tm ramifications. (2) Dm cells are noncolumnar local neurons that do contact photoreceptor layers, whereas Pm cells only have projections in lower medulla layers. Both classes ramify extensively over several columns (Figure S5B). Interestingly, noncolumnar local neurons appear to be pre- and postsynaptic both in photoreceptors layers and in lower medulla layers. This suggests that Dm local neurons perform a first level of integration with Tm cells at the level of photoreceptor layers, whereas this information is further
processed by Pm cells that act at a second level in lower medulla layers.

Therefore, columnar and noncolumnar projection neuron outputs could be modulated by columnar and noncolumnar local neurons, respectively. In the Drosophila antennal lobe, two kinds of local neurons exist: inhibitory local interneurons [37, 38] and a local excitatory population involved in processing projection neuron signals to downstream targets [39, 40]. A similar system might also exist in the medulla.

**Parallel Pathways in Visual Processing**

Whether Tm projection neurons are columnar or noncolumnar, they all arboreal in lower medulla layers (M7–M10). For most Tm cells analyzed, these lower medulla ramifications appear to contain both presynaptic and postsynaptic terminations, suggesting that this region represents a second layer of integration in the color-vision pathway after the direct comparison between R7 and R8 (or between y and p R7/R8).

A class of projection neurons, TmLM, and TmLM only arboreal in lower medulla layers, where they mostly exhibit postsynaptic arborizations. This suggests that they play a role as third-order neurons that collect more elaborate visual information already integrated by other Tm cells with ramifications in photoreceptor layers and presynaptic endings in lower medulla layers and then carry this processed visual information to downstream targets.

**Conclusions**

Our observations have allowed us to reconstruct the organization of the visual circuit in Drosophila. Generating single-cell clones allowed us to decipher many of the intricacies of this pathway and to propose general rules of color-vision processing in the medulla and transmission to downstream targets in the deeper optic lobes. Additionally, we have identified Ga4 lines with very restricted expression patterns in neuronal subtypes in the medulla. Future electrophysiological and behavioral experiments using these and additional Ga4 lines will help reveal the exact function of these optic lobe cells in these complex circuits and to reach a better understanding of the mechanisms that govern the physiology of vision both in invertebrates and vertebrates.

**Experimental Procedures**

**Fly Stocks and Clonal Analysis**

The following lines were used for the study and are expressed in patterns described in the text. These lines are available: 27b-Ga4, 192a-Ga4, 688a-Ga4, 782c-Ga4, 1118-Ga4, 1675a-Ga4, 2135a-Ga4, 2402a-Ga4. The entire piggyBac enhancer trap screen will be published elsewhere (A. Celik, J.M., T. Tabata, and C.D., unpublished data). The other lines, ap<sup>med1</sup>-Gal4, c699-Ga4, dll<sup>122</sup>-Gal4, ey<sup>red</sup>-Gal4, otf-Ga4, ap<sup>Kride-1</sup>-Gal4, glass-lacZ, UAS-CDE-GFP, UAS-nuGFP, UAS-Dscam17.1-GFP, UAS-Syt-HA, UAS-tau-LacZ, and UAS-DsRed, are available in the Bloomington stock center or from the National Center for Research Resources, National Institutes of Health.

**Cell Counting in the Medulla**

We established the percentage of cells in the medulla cortex expressing each Ga4 by comparing the number of cells coexpressing GFP and Elav. The number of positive neurons in each bin was determined as the average percentage of cells in a 9025 μm<sup>2</sup> area (n = 10 brains per genotype) using a 20x immersion lens.

**Immunocytochemistry**

Flies were raised on standard medium at 25 °C. Adult brains were dissected in cold PBS and fixed in 4% PFA for 20 min. Samples were incubated in a cocktail of primary antibodies diluted in 0.3% PBST (Triton X-100 in PBS) overnight at room temperature. Primary antibodies used were as follows: guinea pig anti-DII (1/3000 [25]), mouse anti-CaB10 (1/50, DSHB), mouse anti-anti-Gal (1/500, Promega), mouse anti-Elav (1/50, DSHB), rabbit anti-anti-DII (1/20,000, Cappel), rabbit anti-Elav (1/1000, Clontech), rabbit anti-Anti-GFP (1/1000, Molecular Probes), rat anti-ELA (1/200, Roche), rat anti-DNCad (1/25, DSHB), and sheep anti-Anti-GFP (1/1000, Biogenes). Brains were washed three times for 5 min in PBS and then incubated in secondary antibodies diluted in 0.3 PBST for 3 hr. Secondary antibodies (Molecular Probes) were used as follows: goat anti-rabbit Alexa488 (1/I000), donkey anti-sheep Alexa488 (1/I000), donkey anti-rabbit Alexa555 (1/I000), goat anti-rat Alexa555 (1/500), goat anti-guinea pig Alexa655 (1/500), and donkey anti-mouse Alexa647 (1/200). After washing overnight, brains were mounted in Vectashield (Vector Labs).

**Supplemental Data**

Five figures are available at http://www.current-biology.com/cgi/content/full/18/8/812/DC1/.

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**References**