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Gene regulatory networks during the development of the *Drosophila* visual system

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Abstract

The *Drosophila* visual system integrates input from 800 ommatidia and extracts different features in stereotypically connected optic ganglia. The development of the *Drosophila* visual system is controlled by gene regulatory networks that control the number of precursor cells, generate neuronal diversity by integrating spatial and temporal information, coordinate the timing of retinal and optic lobe cell differentiation, and determine distinct synaptic targets of each cell type. In this chapter, we describe the known gene regulatory networks involved in the development of the different parts of the visual system and explore general components in these gene networks. Finally, we discuss the advantages of the fly visual system as a model for gene regulatory network discovery in the era of single-cell transcriptomics.

Keywords

Gene regulatory network; fate specification; neural diversity; *Drosophila* visual system

Introduction

Cell diversity in the nervous system has long fascinated generations of scientists. Back in the nineteenth century, the pioneering works of Camillo Golgi and Santiago Ramón y Cajal spearheaded a series of studies on how neurons differ in shape, position, and targeting properties (Golgi, 1885; Ramón y Cajal, 1911). The nervous system is so sophisticated that even after more than one hundred years of vigorous interrogation, there are still many unknown types of neurons to be discovered (Boldog et al., 2018), which makes one wonder what underlies this enormous diversity.

In vertebrates and invertebrates, the nervous system originates from a small population of stem cells that seem homogenous in the embryo. The stem cells then generate a wide array of neuron types based on their spatial location and the time when the neurons are born (Holguera and Desplan, 2018). The specification process requires distinct transcription factors expressed in specific spatial and temporal domains and results in a wide diversity of neurons generated in precisely regulated quantity and location (Doe, 2017; Jessell, 2000; Li et al., 2013a; Shirasaki and Pfaff, 2002). Each type of neuron has a stereotypical pattern of innervation, sends and receives signals from a unique set of synaptic partners, and often has a distinctive mode of firing. Researchers have used reverse genetics to identify many of the genes that are involved in the establishment of features like guidance or selection of targets (Kolodkin et al., 1993; Kolodziej et al., 1996; Messersmith et al., 1995; Mitchell et al., 1996;

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Seeger et al., 1993). Furthermore, recent advances in technology have allowed electrophysiological features and gene expression to be systematically compared and have demonstrated that similarity in gene expression correlates with similarity in firing patterns (Cadwell et al., 2016; Fuzik et al., 2016). Starting from either the developmental process or the terminal features of neurons, previous studies have broadened our knowledge on how neurons obtain their identities and morphological and physiological features. Nonetheless, how taking a developmental identity leads to the establishment and maintenance of specific terminal features remains largely unknown.

Understanding regulatory genes and the regulatory networks in which they are embedded would make it possible to explore the combinatorial regulation of such terminal features. A complete gene regulatory network at different times during development would encompass not only the sum of the relationships between each regulator and its target genes but also the interactions among all the regulators and effectors that are involved in a process. The construction of a gene regulatory network would provide insight into how the genes important for the morphological and functional features that we use to define a cell type are regulated such that they are expressed specifically and in a timely manner. With more information about regulatory interactions, we could examine the properties that emerge at the level of control loops, for example, how robustness to disruption is achieved beyond simple redundancy.

The visual system of *Drosophila* is an ideal platform to learn about gene regulatory networks and to bridge the gap between development and terminal features that establish diversity in the nervous system. First, the neurons of the visual system have been studied extensively for decades, and as a result, the morphology and the choice of neurotransmitter of a large proportion of the neurons are characterized (Fischbach and Dittrich, 1989; Raghu et al., 2013; Raghu and Borst, 2011; Varija Raghu et al., 2011). Secondly, the high-resolution connectivity of most neuropils within the optic lobe has been actively determined by electron microscopy (Rivera-Alba et al., 2011; Shinomiya et al., 2019; Takemura et al., 2017, 2015, 2013). These known features that distinguish different types of neurons are invaluable for the discovery of gene regulatory networks, as changes in many of these features can be assessed following perturbations of potential regulators, making it easier to build a predictive model that links regulators to function. Finally, powerful genetic tools in *Drosophila* allow manipulation of the potential regulators in a timely and versatile fashion. For example, previous investigations in the developing optic lobe have identified transcription factors that are limited to specific spatial domains or temporal windows, and precisely timed perturbation of these transcription factors have demonstrated that they are required for the proper development of distinct types of neurons in the optic lobe (Erclik et al., 2017, 2008; Gold and Brand, 2014; Li et al., 2013b; Suzuki et al., 2016).

Traditionally, gene regulation is discovered gene-by-gene, uncovering individual regulators for a target gene, or alternatively, the targets of a regulator are discovered by direct binding to the target sequences or by correlated changes upon perturbation of the regulator. This strategy enables a detailed but highly focused understanding of parts of larger gene regulatory networks. Recently, with insights from previous research and advances in high-throughput profiling of gene expression, systematic inference of gene regulatory networks

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has provided another strategy to investigate broad regulations (Reviewed by Vân Anh Huynh-Thu, 2018). This strategy holds the promise to discover novel regulatory logic that might ultimately allow us to integrate what we have learned previously from single genes into a complete network.

This review focuses on the regulators in the gene regulatory network during the development of the visual system of *Drosophila* and discusses the promises that the fly visual system holds as a platform for systematic gene regulatory network discovery and inference.

Design principles of the gene regulatory networks (GRNs) in the *Drosophila* visual system

Development is a process of gradually specifying various cell types that serve different functions from a small population of stem cells and organizing the specified cells to form a functional entity. The process is precisely regulated in number and in time. For example, neurons in the *Drosophila* optic lobe are generated in a fixed ratio to the number of unit-eyes, and the specification of the neurons happens in a time window from the third larval instar to early pupation. Precision and robustness of the developmental processes are critical for organisms to function properly and are made possible by sophisticated interactions among genes that regulate the timing and amount of the expression of other genes.

A gene regulatory network is the sum of the interactions between genes in time and space and can be further partitioned into interconnected subnetworks that serve specific functions. In the development of the visual system of *Drosophila*, five subnetworks are shared across the visual system but wired differently to fit the specific needs of each region:

1. Tissue commitment subnetwork: An early subnetwork is activated during embryonic development and determines which tissue or organ is formed. Activation of the subnetwork is often sufficient for inducing ectopic formation of the corresponding tissue.
2. Progenitor expansion subnetwork: After establishing tissue identity, a progenitor subnetwork makes sure the tissue is formed in the proper size and delays further differentiation from happening until receiving proper extrinsic cues.
3. Patterning subnetwork: Along with the expansion of progenitors, the tissue is compartmentalized by a subnetwork that contains spatial information and modulates the progenitor subnetwork. Compartmentalization allows different cell types to be generated and organize the developing tissue according to the body plan.
4. Differentiation subnetwork: The differentiation subnetwork integrates multiple extrinsic cues to initiate specification in an accurately timed fashion and results in detailed functional subdivision of cell types.
5. Terminal specification subnetwork: A subnetwork that involves a temporal series of regulators underlies the specification of different neuron types. Different cell

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types are specified according to the integration of the temporal information and the spatial information from the compartmentation subnetwork.

The specification process results in the initiation of the subnetworks that establish the terminal features that make each neuron type distinct.

The eye

The *Drosophila* eye has a highly repetitive structure, consisting of approximately 800 unit-eyes called ommatidia. Each ommatidium contains eight photoreceptor cells, four cone cells, and pigment cells. The *Drosophila* eye originates from the eye imaginal disc, and previous studies of its development have uncovered a versatile gene regulatory network that is modulated by various extrinsic cues (Reviewed in Roignant and Treisman, 2009; Treisman, 2013). Many of the design principles of GRNs in the visual system of *Drosophila* were discovered in the eye and were later shown to apply to other parts of the visual system.

The eye disc GRN

At the root of the eye development network are Twin of Eyeless (Toy) and Eyeless (Ey), the *Drosophila* Pax-6 homologues. Toy and Ey regulate the tissue commitment subnetwork of the eye and are expressed in the eye disc during embryonic stages that define eye disc progenitors, and their ectopic expression in heterologous imaginal discs induces ectopic eye formation (Czerny et al., 1999; Halder et al., 1995). At the first larval instar, Homothorax (Hth) expression begins, and Hth, Ey, Teashirt (Tsh), and Toy form the progenitor expansion subnetwork that maintains proliferation of the eye disc progenitor cells and suppresses the differentiation subnetwork (Bessa et al., 2002; Lopes and Casares, 2010; Zhu et al., 2017).

The developing eye is compartmentalized into dorsal and ventral compartments. The dorsal compartment is defined by the expression of Pannier (Pnr) and its target Wingless (Wg) at the dorsal margin of the eye disc. Wg signaling initiates the patterning subnetwork to delineate the dorsal and ventral compartments by activating the transcription factors Araucan and Mirror encoded by the Iroquois complex (Iro-C) in the dorsal compartment (Cavodeassi et al., 1999; Maurel-Zaffran and Treisman, 2000). Dorsoventral compartmentation also prepares the extrinsic cues that are required for differentiation. Fringe (Fng), a glycosyl transferase, is expressed in the ventral compartment and modifies Notch: Glycosylated Notch does not respond to its ventrally expressed ligand Serrate but responds to dorsally expressed Delta (Dl). The selective sensitivity to Dl ensures that Notch signaling is only active at the boundary between dorsal and ventral compartment (Brückner et al., 2000; Cho and Choi, 1998; Domínguez and De Celis, 1998; Panin et al., 1997; Papayannopoulos et al., 1998) (Figure 1A).

Notch signaling at the dorsoventral midline is required to initiate the morphogenetic furrow at the posterior margin of the eye and terminal specification by promoting proliferation that brings posterior cells out of reach of Wg (see next section) (Kenyon et al., 2003; Kumar and Moses, 2001).

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The GRN of the morphogenetic furrow

At early third larval instar, Hedgehog (Hh) and Decapentaplegic (Dpp), two secreted signaling molecules, are expressed at the posterior margin of the eye disc before differentiation starts (Baker et al., 2018; Borod and Heberlein, 1998; Pignoni et al., 1997). Dpp signaling is induced by Hh signaling and suppresses the expression of Hth.

Retinal differentiation is a progressive process and is initiated when Hh signaling induces a dorsoventral stripe of Dpp expressing cells at the posterior margin, termed the morphogenetic furrow (MF) (Figure 1A, lower panel). Dpp signaling from the MF acts at long distance on cells anterior to it and initiates the differentiation subnetwork, forming the pre-proneural zone (Bessa et al., 2002; Greenwood and Struhl, 1999). Dpp signaling allows Ey to activate Sine oculis (So) and Eyes absent (Eya) in the posterior eye disc at the second larval instar (Curtiss and Mlodzik, 2000; Halder et al., 1998; Heberlein et al., 1995; Lopes and Casares, 2010; Niimi et al., 1999; Ostrin et al., 2006) (Figure 1A, lower panel). Eya and So are required to maintain the expression of Dpp, forming a positive feedback loop, and to directly activate the expression of Daschund (Dac) (Pappu et al., 2005; Pignoni et al., 1997). Eya, So, and Dac form the subnetwork that initiates differentiation and suppresses the progenitor subnetwork (Chen et al., 1997)(Figure 1A, upper panel). Within the MF, short-acting Hh signaling from the posterior margin of the eye disc cooperates with Eya, So, and Dac and activates the expression of a proneural gene, Atonal (Ato) (Borod and Heberlein, 1998; Tanaka-Matakatsu and Du, 2008) (Figure 1A, lower panel). The expression of Ato marks the beginning of specification of R8 photoreceptors and the recruitment of photoreceptor preclusters that later develop into individual ommatidia (Jarman et al., 1994). Differentiating photoreceptors begin to express Hh. Hh signaling from these differentiating photoreceptors reaches the pre-proneural zone and induces Dpp expression, moving the MF anteriorly. Dpp signaling from the moving MF induces a new pre-proneural zone (Borod and Heberlein, 1998). The speed of MF propagation is modulated by the interaction of extrinsic cues to prevent ectopic or precocious differentiation of photoreceptors and to allow the development of a properly sized and patterned eye. For example, Wg signaling from the anterior eye disc suppresses the expression of Ato and slows down MF propagation, while the expression of Wg is suppressed by Dpp signaling (Cadigan et al., 2002; Hazelett et al., 1998). In the pre-proneural zone, Dpp signaling modulates the speed of MF propagation by inducing Hairy, a repressor of Ato, and expression of Delta in the MF suppresses Hairy to allow differentiation to proceed (Baonza and Freeman, 2001; Brown et al., 1995; Greenwood and Struhl, 1999) (Figure 1A, upper panel).

The GRN specifying photoreceptor fate

Within the morphogenetic furrow, the regulation of Ato governs the initiation of photoreceptor preclusters by specifying the R8 photoreceptors that provide the signals required for the sequential specification of all other photoreceptors. Ato is first expressed at a basal level in a dorsoventral stripe in the MF and then resolves into cell clusters containing approximately 15 cells (Dokucu et al., 1996) (Figure 1A and 1B). Within each cluster, only one cell keeps expressing Ato and is specified as an R8 photoreceptor while the other cells lose Ato. The R8 cell expresses Dl and Scabrous (Sca) to inhibit Ato expression in the other

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cella via Notch-mediated lateral inhibition (Baker et al., 1996, 1990; Chen and Chien, 1999; Dokucu et al., 1996; Lim et al., 2008).

R8 is responsible for sequentially triggering the differentiation of other cells in an ommatidium via Notch and EGFR signaling (Baker and Yu, 1998; Jarman et al., 1994; Tio et al., 1994; Tio and Moses, 1997). Ato in R8 cells activates Rhomboid (Rho), which cleaves Spitz (Spi), a secreted EGF ligand that activates EGFR signaling in surrounding non-R8 cells (Baonza et al., 2001)(Figure 1B). EGFR signaling triggers two non-specified cells to express Rough (Ro), a suppressor of R8 fate, and to differentiate into R2 and R5. Ro activates the expression of Rhomboid in R2 and R5, thus increasing EGFR signaling in neighboring cells (Freeman, 1996; Freeman et al., 1992; Kimmel et al., 1990; Pepple et al., 2008; Tomlinson et al., 1988; Yogev et al., 2008). This higher EGFR activity specifies two more cells as R3 and R4 cells that express Seven-up (Svp) (Mlodzik et al., 1990). Svp and EGFR signaling suppress R7 fate in both R3/4 and R1/6 cells (see below)(Begemann et al., 1995). Within the R3 and R4 precursors, the cell receiving a polarizing signal from the poles of the eye via Frizzled (Fz) becomes R3 and expresses Dl that specifies its neighboring precursor as an R4 cell via Notch signaling (Fanto and Mlodzik, 1999)(Figure 1B). R8, R2, R5, R3, and R4 form a photoreceptor precluster. EGFR signaling is necessary and sufficient for maintaining G1 arrest in this precluster, while promoting mitosis in the surrounding unspecified cells, initiating the "second mitotic wave" (Baonza et al., 2002; Baonza and Freeman, 2005; Domínguez et al., 1998; Firth and Baker, 2005; Yang and Baker, 2003). The unspecified cells divide once more to generate enough cells to form the remaining ommatidial cells. Cells closest to the precluster receive EGF signaling and start to differentiate. The first two specified cells express Svp and Delta and differentiate to R1/R6, while the third cell is specified by Notch from R1/R6 and the Bride of sevenless (Boss) local signal from R8. This cell becomes an R7 inner photoreceptor that expresses Prospero (Pros) to suppress the R8 fate and consolidate the R7 fate (Cooper and Bray, 2000; Karpilow et al., 1996; Miller et al., 2009, 2008; Tomlinson et al., 2011; Tomlinson and Struhl, 2001) (Figure 1B). Finally, cone cells and pigment cells are specified by EGFR and Notch signaling without Boss/Sev signaling that cannot reach the cells outside of the photoreceptor cluster.

The GRN that determines Photoreceptor terminal features: Ommatidial subtypes

Ommatidia contain two types of photoreceptors. The outer photoreceptors R1-R6 are involved in motion detection and dim light vision. They express the broad-spectrum Rhodopsin Rh1. The inner photoreceptors R7 and R8 are involved in color discrimination (Yamaguchi et al., 2010, 2008). Ommatidia can be separated into subtypes based on the Rhodopsins expressed in their inner photoreceptors: pale and yellow ommatidia are distributed stochastically in the retina. In yellow ommatidia (65%), R7 expresses UV-sensitive Rh4, and R8 expresses green-sensitive Rh6; in pale ommatidia, R7 expresses UV-sensitive Rh3, and R8 expresses blue-sensitive Rh5. In the dorsal third of the retina, R7s in yellow ommatidia co-express the UV Rhodopsins Rh3 and Rh4, perhaps for improved UV detection for solar orientation, while R7s in pale ommatidia express normally Rh3 alone (Hardie, 1985; Mazzoni et al., 2008). In a single row of ommatidia at the dorsal rim area, both R7 and R8 express Rh3 and are specialized in detecting the vector of polarized UV light for navigation (Weir and Dickinson, 2012; Wernet et al., 2012, 2003). In outer

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photoreceptors, Sine oculis activates the expression of the transcription factor Glass (Gl) that links retina patterning to the morphogenesis of the rhabdomere and the expression of Rhodopsins (Bernardo-Garcia et al., 2016; Moses et al., 1989). Gl directly activates the expression of Rh1, along with Orthodenticle (Otd) and Pph13 (Ellis et al., 1993; Mishra et al., 2010). Otd activates Defective proventriculus (Dve) at a high level that suppresses inner photoreceptor Rhodopsins. In contrast, Spalt (Sal) is expressed in inner photoreceptors and suppresses the default expression of Rh1 and Dve, thus allowing different combinations of Rhodopsins to be expressed in yellow and pale ommatidia (Cook et al., 2003; Mollereau et al., 2001) (Figure 1C). The ratio of yellow and pale ommatidia is controlled by the stochastic activation of the transcription factor Spineless (Ss) that specifies yellow R7s (Johnston et al., 2011; Thanawala et al., 2013; Yan et al., 2017). In yellow R7s, Ss forms a heterodimer with Tango (Tgo) to directly activate Rh4 and to activate Dve at a low level that suppresses Rh3 (Johnston et al., 2011; Thanawala et al., 2013; Yan et al., 2017). In pale R7s lacking Ss, Otd and Sal activate the expression of Rh3. In the yellow R7s of the dorsal third, the expression of Iro-C genes overcomes the suppression of Rh3 by Dve while still maintaining expression of Ss at a lower level (Mazzoni et al., 2008; Thanawala et al., 2013), and as a result, dorsal third yellow R7s express both Rh3 and Rh4. Finally, in the dorsal rim area (DRA), Wingless signaling and IroC activate Homothorax (Hth) to suppress Ss, and consequently Rh4. As a result, DRA R7s only express Rh3 (Wernet et al., 2006, 2003). Interestingly, R8s in the DRA also express Hth and Rh3 (see below and Figure 1D).

The expression of Rhodopsins in R8 is coordinated with that of its partner R7. In a *sevenless* mutant that lacks R7s, most R8s express Rh6 and resemble yellow R8s, suggesting that pale R7 provides a signal that allows for the expression of Rh5 in R8s (Chou et al., 1999; Papatsenko et al., 1997). Pale R7s activate a bistable loop in R8s consisting of two genes cross-regulating each other, Warts (Wts), a tumor suppressor kinase, and Melted (Melt), a growth regulator. Sens, a critical transcription factor for specifying R8, promotes by default the expression of Wts, which represses Melted and activates Rh6. In pR8, Activin and BMP signals from pale R7 activate Melt, which suppresses Wts and allows Otd and Yki to activate Rh5 (Jukam et al., 2013; Jukam and Desplan, 2011; Wells et al., 2017). In the dorsal third yellow ommatidia that express both Rh3 and Rh4 in R7, R8s still express Rh6, suggesting that the Activin signal from R7 is suppressed by Ss, independently from the presence of Rh3. In the dorsal rim area, Wingless signaling and the dorsal IroC genes cooperate to induce the expression of Hth not only in R7 but also in R8. In these R8s, Hth suppresses Sens and consequently Rh6 while Hth, Extradenticle, Otd, and Sal act cooperatively to activate Rh3 that is therefore expressed in both R7 and R8. By expressing Rh3 in both R7 and R8, DRA ommatidia are not involved in color vision but instead compare the angle of light polarization (Wernet et al., 2003; Wernet and Desplan, 2014) (Figure 1D).

The GRN that determines photoreceptor terminal features: Axon targeting

Immediately after specification, the photoreceptors extend their axons to different layers of the optic lobe. Then, much later, they form the rhabdome, the light-gathering structure that contains the Rhodopsins that detect different spectra of light (Mollereau et al., 2001). Outer photoreceptors (R1–6) detect motion; they express the broad-spectrum Rh1 and terminate their axons in the lamina, the first neuropil of the optic lobe (see Figure 2A for the anatomy

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of the optic lobe). Inner photoreceptors (R7 and R8) express distinct Rhodopsins that are sensitive to different wavelengths of light and are involved in color vision (Jukam et al., 2008; O'Tousa et al., 1985). R7 and R8 terminate their axons in the medulla part of the optic lobe (Reviewed in Morante and Desplan, 2004). Glia-derived signals in the lamina are critical for axons from outer photoreceptors to terminate in the lamina while the axons from the inner photoreceptors extend to the medulla (Poeck et al., 2001). Several genes are required for proper targeting of outer photoreceptors. For example, the loss of Off-track, a receptor tyrosine kinase, in outer photoreceptors results in axons wrongly terminating in the medulla (Cafferty et al., 2004). Similarly, Misshapen, a serine/threonine kinase, is expressed in photoreceptor cells under the control of Glass, interacts with the SH2/SH3 adaptor protein Dreadlocks, and is required for proper axon termination in the lamina (Ruan et al., 2002, 1999; Treisman et al., 1997).

The inner photoreceptor R8 exhibits stepwise targeting: during mid-pupation, two genes, Gogo and Flamingo (Fmi), are required to guide the R8 axons into the medulla. Gogo is mainly expressed in R8s and the optic lobe, while Flamingo is expressed unevenly in the growth cones of R1–6 but not the optic lobe (Lee et al., 2003; Tomasi et al., 2008). The chemotropic guidance molecule Netrin is expressed in the R8 target layer and is recognized by its R8-expressed receptor, Frazzled, that facilitates targeting specificity (Timofeev et al., 2012).

The fate-specifying factors could also be involved in the regulation of axon targeting. For example, in R8s, Sens cooperates with Otd to regulate the targeting subnetwork in R8s. Otd is required to activate the expression of Gogo and Fmi (Mencarelli and Pichaud, 2015), and along with Otd, Sens activates Capricious, a R8-specific cell adhesion molecule required for its targeting. In R7s, NF-YC suppresses the R8 axon targeting subnetwork (Morey et al., 2008).

Although in most cases, it is not yet known how regulators of axon targeting are linked to the regulatory networks that specify the fate of the photoreceptors, a few regulators that coordinate photoreceptor axon targeting have been identified (Hoi et al., 2016; Kniss et al., 2013; Kulkarni et al., 2016; Oliva and Sierralta, 2010). For example, Brakeless, a transcriptional repressor expressed in photoreceptors, is required for proper targeting of the outer photoreceptors to the lamina by repressing Runt, which is only expressed in inner photoreceptors. Mis-expression of Brakeless or Runt in photoreceptors does not alter the expression of the fate-specifying factors like Pros or Rough, suggesting this cross-regulation happens downstream of or parallel to the fate-specifying factors. However, mis-expression of Runt in R1-R6 leads to their ectopic projection to the medulla (Kaminker et al., 2002; Rao et al., 2000; Senti et al., 2000).

GRNs in the eye: A brief summary

Gene regulation during fly retina development has been extensively studied and provides the likely framework that times and coordinates the specification of terminal features. The first step during embryonic retina development is Ey and Toy promoting the potency of the progenitor cells to form an eye. During the proliferation of retinal progenitors, extrinsic cues modulate the progenitor gene regulatory network, first to compartmentalize the eye disc into

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dorsal and ventral compartments, and later to trigger differentiation. Dpp, Notch, and Hh signaling pathways activate the retinal determination genes, Eya, So, and Dac, which initiate cell-type specification by promoting a sequential specification subnetwork regulated by Ato in the photoreceptor preclusters. During terminal differentiation, the Rhodopsin subnetwork regulated by Glass and Spalt is activated to define motion- versus color-sensing photoreceptors. Ommatidial subtypes are defined by a stochastic subnetwork regulated by Ss and by signaling by Iro-C and Wg. Less is known about the subnetwork that determines axon targeting. Some fate-specifying factors, like Sens, is shown to also play a central role in axon targeting, while it is not yet known if the regulators that distinguish innervation of lamina versus medulla is also under the factors that secure the identity of each photoreceptor cell, like Ro, Pros, and Svp, or alternatively, if the innervation and targeting regulators are directly under the control of retinal determination genes and form their own subnetwork.

The GRN that specifies lamina neurons in response to photoreceptor signals

Outer photoreceptors R1-R6 innervate the lamina part of the optic lobe. The neuroepithelium that gives rise to the optic lobe proliferates and segregates into two domains at the first larval instar: The Fas3-expressing inner proliferation center (IPC) that gives rise to lobula plate neurons, and the Outer Proliferation Center (OPC) that does not express Fas3 (Gold and Brand, 2014; Tayler et al., 2004)(Figure 2A). The inner part of the OPC crescent will become the lamina, the part of the optic lobe first involved in motion vision, while the outer part of the crescent will give rise to the medulla (Huang et al., 1998; Huang and Kunes, 1998, 1996). Lamina neurons develop synchronously with the MF that moves through the eye and critically depend on innervation by photoreceptors R1-R6. Axon bundles of outer photoreceptors extend to the optic lobe neuroepithelium and induce proliferation of their target field and differentiation into five types of lamina neurons, L1-L5. The progenitor expansion subnetwork for lamina neurogenesis includes Hth, Eya, and So, which promotes cell proliferation and suppresses a differentiation subnetwork (see below). Photoreceptor axons secrete several signaling molecules when they arrive in the developing OPC. The neuroepithelial cells in the inner OPC crescent respond to Hedgehog produced from photoreceptor axons (Huang and Kunes, 1998, 1996; Selleck and Steller, 1991) (Figure 2C). Hedgehog signaling mediates the transition from neuroepithelium to lamina precursor cells (LPCs) by rewiring the progenitor expansion subnetwork: Eya and So activate Dac in the presence of Hh signaling, which in turn suppresses the expression of Hth. Therefore, the precursor subnetwork still promotes proliferation but also allows the differentiation subnetwork to be activated (Piñeiro et al., 2014). Down-regulation of Hth allows the expression of the transcription factor Single-minded (Sim), which activates the expression in LPCs of the cell adhesion molecule Hibris that interacts with Roughest in photoreceptors, thus allowing the lamina precursor cells to be recruited to photoreceptor axon bundles to assemble into lamina cartridges (Piñeiro et al., 2014; Sugie et al., 2010; Umetsu et al., 2006). Glial precursors are recruited earlier to extending photoreceptor axons than the and differentiate in response to signals secreted by the photoreceptor axon bundles (Perez and Steller, 1996; Winberg et al., 1992). Glia precursor cells originate from the optic stalk and migrate to the developing lamina along photoreceptor axons that produce FGF (Choi and

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Benzer, 1994; Chotard and Salecker, 2007). Photoreceptor axon-derived FGF is required for the differentiation of the wrapping glia that wrap the bundle of photoreceptor axons as they progress towards the lamina (see below) (Franzdóttir et al., 2009). Photoreceptor axons also secrete an EGF ligand (Spitz) that is specifically processed and secreted in the axons of photoreceptors and induces the differentiation of LPCs into the five types of lamina neurons, L1-L5 (Huang et al., 1998; Huang and Kunes, 1998, 1996; Yogev et al., 2008). However, this effect is indirect and is in fact mediated by wrapping glia that are the cells that receive the EGF signal from photoreceptor axons and secrete in response Insulin-like peptides as they extend down the photoreceptor axon bundle into the developing lamina. Insulin-like peptides are required for the sequential specification of lamina neuron L1-L5 (Fernandes et al., 2017; Rossi and Fernandes, 2018) (Figure 2B). Therefore, lamina precursor cells are produced and differentiate into distinct cell types in response to photoreceptor innervation. Supernumerary cells recruited to the cartridges are eliminated by apoptosis, and the lamina does not form at all in the absence of photoreceptors (Huang et al., 1998; Huang and Kunes, 1998, 1996).

Lamina neurons then innervate different layers in the medulla. A number of transcriptional regulators and other factors are required for precise layer selection in a cell type-specific manner. N-Cadherin, Acj6, and Earmuff are examples, but how the regulation of these genes is coupled to cell type specification remains unclear (Certel et al., 2000; Nern et al., 2008; Peng et al., 2018).

In short, the gene regulatory network of lamina neurogenesis responds to extrinsic cues provided by photoreceptor axon bundles. The tissue commitment gene regulatory subnetwork that determines whether the OPC neuroepithelium would give rise to LPCs or to medulla neuroblasts is still unclear. One candidate regulator is Tailless (Tll), which is broadly expressed in OPC neuroepithelium at early first larval instar and later becomes restricted to the neuroepithelium lateral to the lamina furrow to generate lamina neurons. Tll is required for lamina precursor cell specification (Guillermin et al., 2015).

The progenitor expansion network responsible for promoting proliferation and suppression of premature lamina neuron differentiation includes Hth, Eya, and So, while Hh signaling from photoreceptor axons rewires the progenitor subnetwork by inducing Dac, which suppresses Hth and allows the activation of the differentiation subnetwork in response to glia-derived insulin-like peptide. The division and recruitment of LPCs to photoreceptor axons that sequentially arrive in a posterior-to-anterior order ensure retinotopy along the anterior-posterior axis.

The terminal specification subnetwork that specifies the five lamina neurons L1-L5 remains elusive. There could be an intrinsic transcription factor cascade triggered by the glial derived Hh signal, and this cascade would allow different cell types to form when the LPCs receive glial-derived signal at different time windows in the cascade; alternatively, the sequential specification of lamina precursor cells could be achieved by their interaction with the extending wrapping glia or by short range signals that are provided by the neurons specified early and tune the fate potential of cells around them, resembling the sequential specification of different photoreceptor subtypes in the eye.

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Temporal and spatial GRNs specify medulla neurons

The medulla is the largest of the optic ganglia, containing more than 40,000 neurons belonging to around 100 different neuron types with distinct morphology and innervation pattern. The medulla neuropil is subdivided into 10 layers (Fischbach and Dittrich, 1989; Morante and Desplan, 2008) and is made of 800 columns corresponding to the 800 ommatidia in the eye. The outer part of the OPC neuroepithelium crescent gives rise to the medulla. It proliferates under the control of a progenitor expansion subnetwork regulated by Eya, So, and Hth until the beginning of the third larval instar (Apitz and Salecker, 2016). Eya, So, and Hth are common regulators in the progenitor expansion subnetwork for neurogenesis of the lamina and medulla. A comparison of the progenitor expansion subnetworks in the retina, lamina, and medulla provides an example of how GRNs are built with a shared design but wired differently for each part of the visual system: Hth is involved in the progenitor subnetwork of the retina, lamina, and medulla and maintains progenitor proliferation, while Eya and So are only required for proliferation in the lamina and medulla but not in the retina, where they are regulators of the specification subnetwork and activate the expression of the proneural factor Ato (Apitz and Salecker, 2016; Bonini et al., 1993). The expansion of the OPC epithelium is also dependent on the expression of Vsx1 (Erclik et al., 2008). During its very early proliferation, the OPC neuroepithelium is compartmentalized along the dorso-ventral axis by a patterning subnetwork containing Vsx, Optix, and Rx, which each define specific compartments and negatively cross-regulate each other, and by signaling molecules that subdivide all compartments (Hh) or only the Rx compartments (Wg and Dpp). Each spatial domain has the potential to generate different neuron types (Chen et al., 2016; Erclik et al., 2017; Evans et al., 2009; Gold and Brand, 2014; Kaphingst and Kunes, 1994).

At the beginning of the third larval instar, a proneural wave begins at the outer edge of the OPC neuroepithelium crescent, in which neuroepithelial cells respond to the combinatorial action of EGFR, Fat/Hippo, JAK/STAT, and Notch signaling to activate the differentiation subnetwork. In the neuroepithelium, EGFR signaling induces the expression of Rhomboid, which processes the EGFR ligand Spi to reinforce EGFR signaling that activates the expression of the Notch ligand Delta (Yasugi et al., 2010). Increasing Delta activates Notch signaling that works with the JAK/STAT and Fat/Hippo signaling pathways to slow down the proneural wave and to maintain the integrity of the optic neuroepithelium (Kawamori et al., 2011; Ngo et al., 2010; Reddy et al., 2010; Wang et al., 2011). During the differentiation into neuroblasts, ever increasing Delta suppresses Notch signaling, presumably by cis-inhibition (Egger et al., 2010), allowing the expression of lethal of scute (L(1)Sc), a proneural transcription factor. As a result, the combinatorial action of extrinsic cues leads to a wave of L(1)sc that specifies the medulla neural stem cells (neuroblasts) that progressively emerge from the neuroepithelium (Egger et al., 2010; Orihara-Ono et al., 2011; Wallace et al., 2000; Weng et al., 2012).

Neuroblasts divide asymmetrically, re-generating a neuroblast and producing a ganglion mother cell (GMC) (Egger et al., 2007). Deadpan (Dpn) is expressed in the neuroblasts to promote self-renewal, while in the ganglion mother cell, L(1)sc, as part of the Achaete-Scute proneural complex, activates Prospero (Pros), which promotes cell cycle exit and thus only

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allows a single division of the GMC to produce two neurons (Choksi et al., 2006; San-Juán and Baonza, 2011; Yasugi et al., 2014)(Figure 3A).

A series of transcription factors are sequentially expressed in the medulla neuroblasts undergoing asymmetric division and are part of the terminal specification subnetwork. Homothorax (Hth), then Eyeless (Ey), Sloppy paired 1 (Slp1), Dichaete (D), and finally Tail-less (Tll). These temporal transcription factors (tTFs) form a temporal sequence as the dividing neuroblasts age. tTFs in this temporal cascade cross-regulate each other. Specifically, Ey, Slp1, and D are required to turn on the next tTF, while Slp1, D, and Tll are required to repress the previous tTF. Different GMCs are produced by each neuroblast at each temporal window. These GMCs undergo Notch-dependent asymmetric division, giving rise to two daughter cells with different fates. For example, the GMC emerging from neuroblasts expressing Hth give rise to a Notch-on neuron that expresses Bsh and Apterous (Ap) and becomes a Mi1 neuron, and a Notch-off cell that expresses Lim3 and Svp and becomes a Pm1, Pm2 or Pm3 neuron, depending on the location of the neuroblasts (see below) (Erclik et al., 2017; Li et al., 2013a; Suzuki et al., 2013) (Figure 3B).

In the current model, medulla neurons can be categorized into two types: Uni-columnar neurons are present as one per medulla column (i.e. 800 neurons of each type) and are likely produced by each neuroblast independently of its spatial position in the neuroepithelium. In contrast, multi-columnar neurons innervate multiple medulla columns and are thus less abundant. The specification of multi-columnar neurons requires the integration of the spatial compartment in which the neuroblast was born and the temporal cascade of tTFs. For example, Pm1/2/3 neurons are multi-columnar and originate from Hth-expressing/Notch-off GMCs. Pm3 neurons come exclusively from the Vsx1-expressing neuroblasts, while Pm1 neurons are generated from ventral Rx-expressing neuroblasts and Pm2 from dorsal Rx neuroblasts (Erclik et al., 2017). The intersection of temporal, spatial, and Notch signaling therefore generates a wide array of neuron types in the medulla.

The neuroblasts at the tip of the OPC neuroepithelium (tOPC), which expresses Rx and Wingless, follow a modified tTF cascade as they sequentially express Distal-less (Dll), Ey, Slp, and D. tOPC neuroblasts at the Dll stage are type 0 and directly differentiate into a single neuron. On the other hand, GMCs from the Ey, Slp, and D window give rise to two cells which differ in Notch signaling. Notch signaling then regulates an apoptotic switch: Notch-on neurons die in the Ey window while it is the Notch-off neurons that die in the Slp and D windows (Bertet et al., 2014) (Figure 3C). Little is known about how the terminal features like the choice of neurotransmitters or the synaptic partners are determined downstream of the specification subnetwork in the medulla, a highly heterogenous ganglion. However, recent advances in single-cell mRNA-seq will allow gene expression to be studied even in very heterogeneous brain tissues. Indeed, transcription factors that are required for the expression of neurotransmitter-related genes were recently identified from a single-cell transcriptome atlas of the *Drosophila* optic lobe (Konstantinides et al., 2018). Further investigation of gene expression in the developing optic lobe are warranted to reveal the gene regulatory networks governing the terminal features like target selection or synapse specificity, which is likely to happen earlier in development (Li et al., 2017).

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The GRNs leading to the formation of the lobula and lobula plate

The inner proliferation center (IPC) neuroepithelium generates distal cells (C2, C3, T2, T2a and T3) and lobula plate neurons (T4 and T5) (Reviewed in Contreras et al., 2019). Anatomically, the IPC neuroepithelium can be subdivided into proximal, surface, and distal domains (p-IPC, s-IPC, d-IPC) (Apitz and Salecker, 2015). The tissue commitment subnetwork that distinguish IPC from OPC and the progenitor expansion subnetwork that maintains the expansion of IPC neuroepithelium have not yet been discovered, but we know that the progenitor subnetwork is distinct from the other regions because Eya and So are not expressed in p-IPC neuroepithelium (Apitz and Salecker, 2016). The p-IPC neuroepithelium is spatially compartmentalized into dorsal and ventral subdomains that express Dpp and flank a central subdomain that expresses Brinker (Brk). Another domain at the ventral tip of the IPC expresses, and is specified by Wg (Apitz and Salecker, 2018), and has a different type of neurogenesis (Filipe Pinto-Teixeira, personal communication).

Dpp signaling from the dorsal and ventral subdomains of the p-IPC triggers the differentiation subnetwork in p-IPC neuroepithelial cells and stimulates their migration from the p-IPC to the d-IPC. The migration requires escargot, a Snail-type transcription factor that mediates epithelial-mesenchymal transition. p-IPC neuroepithelial cells express L(1)sc when they differentiate into migratory progenitors. Migratory progenitors than differentiate into Dpn- or Brk-expressing neuroblasts when they arrive at the lower d-IPC where they intermingle with each other to become d-IPC neuroblasts. The terminal specification subnetwork promotes d-IPC neuroblasts to progress through a series of tTFs as they divide and are pushed from the lower to the upper d-IPC by newly arriving progenitors (Apitz and Salecker, 2015). Young neuroblasts in the lower d-IPC express Ase and Dichaete and generate distal cells (C2, C3, T2, T2a and T3) that express Acj6 or Toy. As neuroblasts age, Ase and Dichaete are down-regulated, while Ato, Tll and Dac are up-regulated. Ato promotes the proliferation of the neuroblasts and later cell cycle exit by inducing the expression of Brat. The Ato-expressing neuroblasts in the upper d-IPC give rise to lobula plate neurons T4 and T5, which express Dac and Acj6 (Apitz and Salecker, 2015; Mora et al., 2018; Pinto-Teixeira et al., 2018). Upper d-IPC neuroblasts undergo two asymmetric divisions: The neuroblasts accumulate Pros asymmetrically, and when they divide, one of the daughter cell inherits cortical Pros while the other expresses Pros right after cell division, producing two Pros-expressing GMCs and then four neurons (Pinto-Teixeira et al., 2018)

T4 and T5 neurons detect local motion and can each be categorized into four subtypes according to the four cardinal directions that they are tuned to detect (front-to-back, a subtype; back-to-front, b subtype; up, c subtype, or down, d subtype) (Maisak et al., 2013). Their diversity arises from the intersection of the spatial compartment they originated from (Dpp or Brk) and from Notch signaling: Horizontal motion detectors T4 and T5 subtypes a and b are generated exclusively from the Brk-expressing domain in the p-IPC neuroepithelium, while vertical motion detector subtypes c and d originate from the Dpp-expressing domains (Apitz and Salecker, 2018; Pinto-Teixeira et al., 2018).

The first Notch-dependent asymmetric division of the upper d-IPC neuroblast decides the directionality of the local motion that the neurons generated detect: For the Brk^{+} p-IPC-

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derived neuroblast, the asymmetric division delineates subtype a (front-to-back motion-sensing neurons) *vs.* b (back-to-front motion-sensing neurons), while, for the Dpp$^+$ p-IPC-derived neuroblast, the division delineates subtype c (upward motion-sensing neurons) *vs.* d (downward motion-sensing neurons). For the second asymmetric division of the GMC, the Notch-off daughter cell becomes a T4 neuron while the Notch-on daughter cell becomes a T5 neuron (Pinto-Teixeira et al., 2018) (Figure 3D).

Many questions about the neurogenesis in the IPC remain unanswered to date. For example, how different types of distal cells are specified is still unknown. It is likely that a similar network is modulated by extrinsic cues and contains subnetworks for neuroepithelium expansion, spatial compartmentation, and tTF transitions to generate different neuron types. T4/T5 neurons present a unique model for studying the subnetworks that define axon and dendrite targeting and synaptic specificity: Although T4/T5 neurons are transcriptionally indistinguishable in the adult (Konstantinides et al., 2018), T4 neurons terminate their dendrites in the medulla, while T5 neurons terminate theirs in the lobula; on the other hand, T4/T5 a, b, c or d subtypes differ in the lobula plate layer in which the neurons project their axons to. Recent single-cell transcriptomic studies have shown that distinct genes are differentially expressed in each T4/T5 subtype during development. These subtype-specific genes are likely to be the effectors of the subnetworks that governs layer selection during neurite targeting (Kurmangaliyev et al., 2019). Further studies are required to uncover the regulators that integrate the inputs from the two Notch-dependent division and Dpp signaling and activate the downstream subnetworks that control dendrite and axon targeting and assemble the motion detection circuit.

Conclusion and perspectives

Gene regulatory networks of the development of the *Drosophila* visual system can be subdivided into interconnected subnetworks, and the interplay of subnetworks underlies the specification of the progenitors to generate the eye or different parts of the optic ganglia, the expansion of the progenitor pool to meet the number of neurons to generate, the compartmentalization of the progenitor pool along body axis to generate different neurons, and the temporal sequence of regulators that specify different types of neurons cooperatively with spatial information from the compartmentalization.

General design principles of the gene regulatory networks of the development of the *Drosophila* visual system could apply to neural development in different species. In some cases, not only the gene but also its function can be evolutionarily conserved across multiple species, so the regulatory network in which a *Drosophila* transcription factor is involved will help learn what its vertebrate orthologue controls. For example, Homothorax (Hth), the key factor of the subnetwork that expands the progenitor pool in the *Drosophila* eye, lamina, and medulla, has several vertebrate orthologues, the MEIS family proteins, which serve similar roles in vertebrates to promote cell proliferation in vertebrate body plan patterning (Bessa et al., 2008). Alternatively, a gene that is conserved across species could function differently, even if it is involved in the same development process. If this is the case, gene regulatory networks in *Drosophila* could still suggest how general developmental processes are structured mechanistically. For example, sequential specification of different cell types from

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neural stem cells is observed in several mammalian systems such as the retina and cortex (Chugh et al., 2014). Although it remains unknown whether the temporal transcription factors are conserved in the brain of fruit flies and mammals, the detailed network structure of temporal transcription factor cascade we learn from *Drosophila* could give hints on the regulators and the topology of the networks that drive the temporal sequence of specification in mammals. Additionally, a comparison of networks that serve similar purposes in *Drosophila* and other species could shed light on how innovations are achieved in evolution. For instance, the difference in progenitor expansion accounts for one of the major differences between the nervous system of human, non-human primates, and other mammals (Boyd et al., 2015; reviewed in Dehay et al., 2015), and the molecular identity of neural stem cells with increased proliferation potential in primates has recently been characterized (Pollen et al., 2015). By comparing the progenitor expansion subnetwork that drives proliferation in different species, we could understand the molecular basis of neocortex expansion.

There are still many missing pieces in the gene regulatory network. In many cases, although the regulators for certain functions have been identified, how these regulators are regulated by the fate specifying transcription factors remains elusive. For example, although we know both the regulators important for the specification of inner photoreceptors and the regulators allowing inner photoreceptors to target the medulla, how these subnetworks interact is still mostly unknown.

Similarly, even if the regulators required for fate specification are identified, the targets of those regulators that define terminal features are often unknown. The spatiotemporal patterning in medulla and lobula plate is a great example in which we know regulators required for fate specification but still do not have a precise idea of what these regulators regulate to allow the neurons to acquire their terminal features.

With the advent of high-throughput single-cell mRNA profiling techniques, computational approaches aiming to uncover regulatory networks systematically from profiling results are being actively developed and give promising results in filling the gaps in the networks. For example, Sens is predicted to be a direct target of Ato in one such study. If Sens were to be a *bona fide* target of Ato, this would explain how R8 fate is maintained after transiently expressed Ato is turned off. It also favors a cascade that is initiated by Ato and later results in the default expression of Rh6, a Rhodopsin specific to R8 photoreceptors and medulla targeting of R8 cells (Aerts et al., 2010; Davie et al., 2018; Mencarelli and Pichaud, 2015).

Existing techniques that infer gene regulation rely on bulk mRNA profiling data from different conditions of samples that are assumed to be homogeneous. The number of samples is often limited, and diversity within the sample is averaged and thus undetectable. The ability of single-cell RNA-seq to distinguish individual cells allows unsupervised clustering of cells according to their states, and the scalability to capture hundreds of thousands of cells makes it easier to model complicated and non-linear relationships between genes. Scalability also enables the capture of continuous processes and the detection of regulators that are transiently expressed. A recent study from Konstantinides et al. used single-cell RNA-seq to predict the regulators of neurotransmitter choices in neurons

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of the developing optic lobe and demonstrated that each of the predicted regulators is only required for the expression of neurotransmitter-related genes in a subset of neuron types. The phenotypic convergence of distinct regulators explains how core regulatory complexes specific for cell types could regulate features that are shared between multiple cell types (Konstantinides et al., 2018).

The advances of experimental techniques bring not only potentials but also challenges for the study of gene regulatory networks: the tremendous number of observations from single-cell RNA-seq experiments is computationally demanding to analyze and requires more efficient algorithms. The sparseness and noisiness warrant refinements of existing analytical tools before being effectively applied. Additionally, the resulting gene regulatory network from inference could be too complicated to interpret and test with experiments.

The *Drosophila* visual system is an excellent model of modern gene regulatory network study. First, our understanding of regulators at different stages of development and the characterization of neural diversity and various terminal features provide a tremendous system to test newly developed analytic tools and to compare the performance of existing tools. Secondly, the number of subnetworks described in previous research limits the number of regulatory relationships to infer and examine in order to bridge the gaps within validated subnetworks. Finally, the powerful genetic tools available in flies allow precisely timed manipulations in specific neuron types and consequently provide the means to examine experimentally complex predicted subnetworks. Leveraging the rapidly evolving techniques and the advantages of the *Drosophila* visual system, we could soon be equipped to answer the long-held outstanding questions: What regulators does it take to define a fate? How is fate linked to connectivity and morphology? Are there other features that differ between neuron types?

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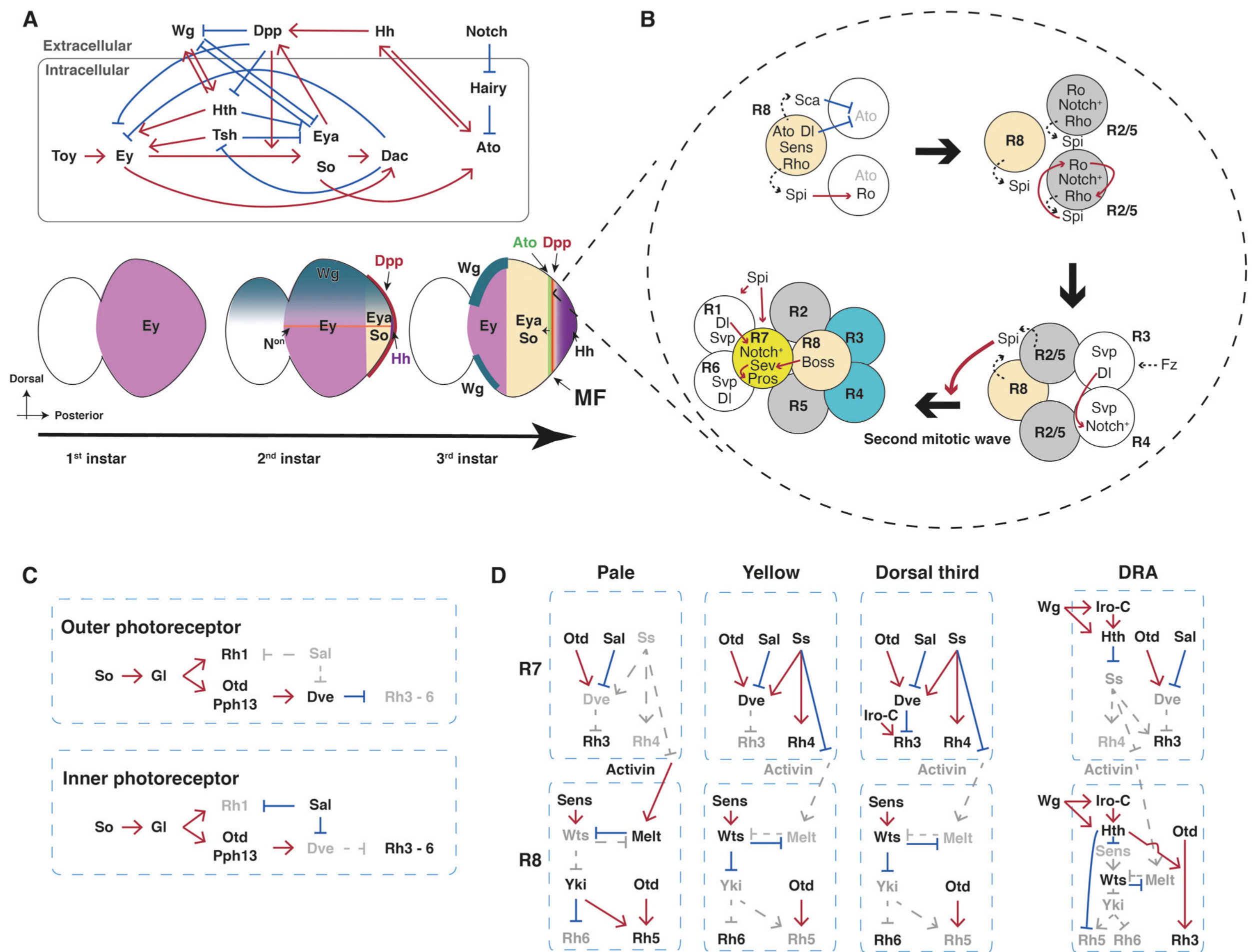

Figure 1. Gene regulatory networks during retinal development

(A) The eye disc progenitor gene regulatory network responds to extrinsic cues to initiate differentiation.

(B) Cross-regulation of fate-specifying transcription factors induced by short-range extrinsic cues from photoreceptors specified earlier in the cluster allows sequential generation of photoreceptors in an ommatidium.

(C) Spalt governs the spectral differences of outer and inner photoreceptors.

(D) A network of transcription factors integrates spatial extrinsic cues and stochastic intrinsic choice to define the ommatidial subtypes that form the retinal mosaic.

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Figure 2. Anatomy of the optic lobe and neurogenesis in the lamina

(A) Anatomy of the developing optic lobe at third larval instar (left panel) and adult optic lobe (right panel). The outer proliferation center generates lamina and medulla neurons, while the inner proliferation center generates distal cells (T2, T2a, T3, C2, C3) and lobula plate neurons (T4/T5). OPC: outer proliferation center; IPC: inner proliferation center; NE: neuroepithelium; NB: neuroblast; LN: Lamina neuron; LPC: Lamina precursor cell; LPN: lobula plate neuron. Yellow arrowheads: The direction of the wave of neurogenesis.

(B) Photoreceptor axon bundles secrete signal molecules to recruit laminal precursors and trigger lamina neuron differentiation. This is mediated by signals secreted by wrapping glia.

(C)Signals from photoreceptor axon bundles trigger the differentiation of lamina precursors and recruit the precursors to form lamina cartridges.

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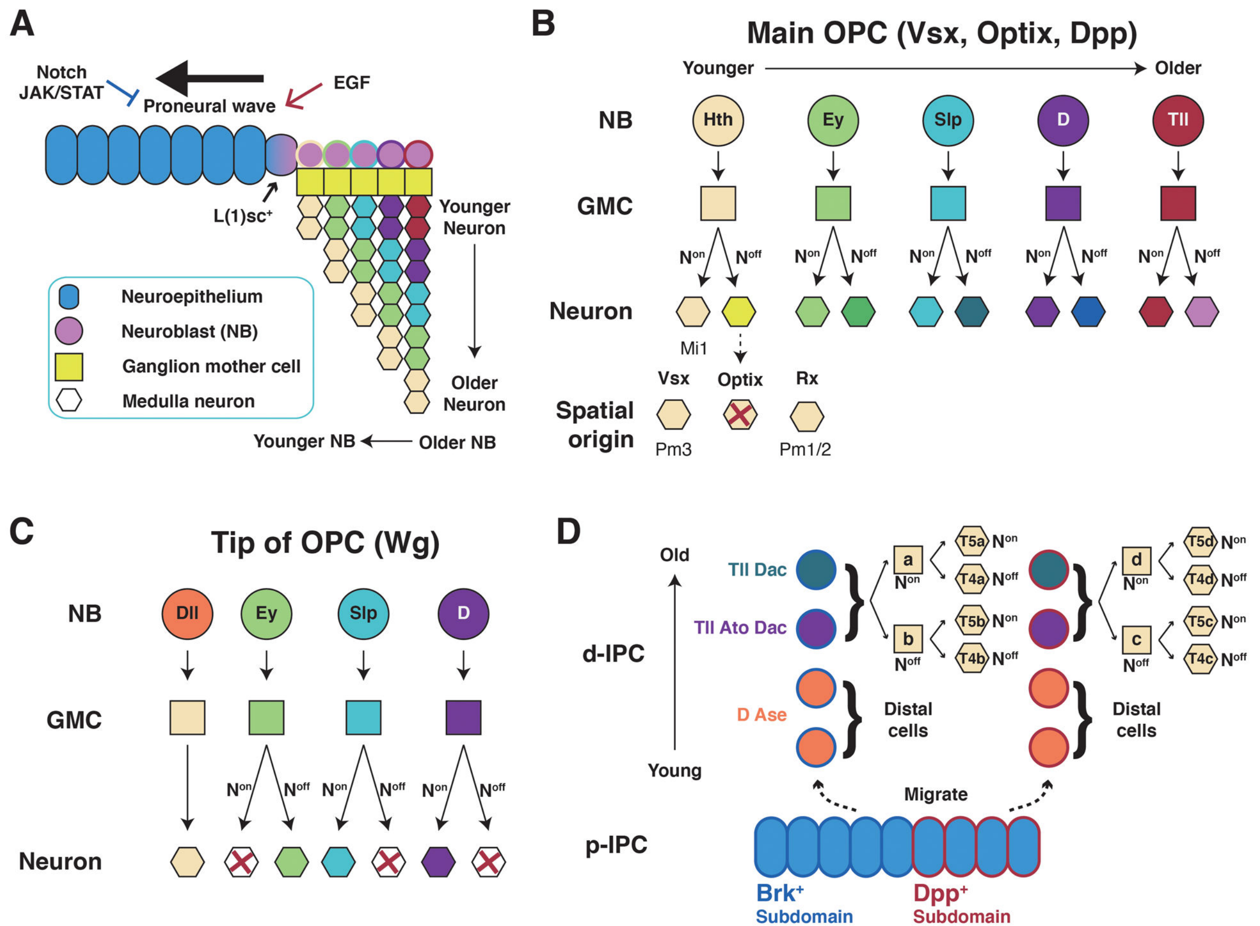

Figure 3. Neurogenesis in the medulla and lobula complex

(A) EGF signaling initiates the proneural wave in the OPC neuroepithelium.

(B) Main OPC neuroblasts from the Vsx, Optix and Dpp domains undergo temporal transition of transcription factors (tTFs) and generate distinct neuron types based on the combination of the tTFs, the domain of neuroepithelium from which neuroblasts originate, and Notch signaling.

(C) The domain of the neuroepithelium that expresses Wingless at the tip of OPC gives rise to tOPC neuroblasts. tOPC neuroblasts generate neural diversity with a cascade of tTFs that is similar but distinct from that of OPC neuroblasts.

(D) Neuroepithelial cells delaminate from the proximal IPC (p-IPC) and migrate to the distal IPC (d-IPC) where they differentiate into neuroblasts. d-IPC neuroblasts undergo a temporal transcription factor transition and first give rise to distal cells and later to T4/T5 neurons. T4/T5 neurons are then specified into four subtypes by two consecutive Notch-dependent divisions.