Cell surface control of the layer specific targeting in the Drosophila visual system

Satoko Hakeda-Suzuki and Takashi Suzuki

Core Division of Advanced Research, Graduate School of Bioscience & Biotechnology, Tokyo Institute of Technology, Nagatsuta 4259, Midori-ku, Yokohama, Kanagawa 226-8501, Japan

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To achieve the precise wiring of axons in the brain required to form a fine architecture, a molecular level interaction between axons and their targets is necessary. The Drosophila visual system has a layered and columnar structure which is often found in the brain of vertebrates. With powerful genetic tools for its analysis, the Drosophila visual system provides a useful framework to examine the molecular mechanisms of axon targeting specificity. The medulla is the second optic ganglion in the Drosophila optic lobe, and is subdivided into ten layers. Among the eight photoreceptor types, R7 and R8 pass through the first optic ganglion lamina and innervate the medulla. In the medulla, R7 and R8 axons grow in a distinct manner to reach their final target layers: M6 and M3, respectively. The axons from R7 and R8 take characteristic steps to extend toward their target layer. In this review, we discuss the formation of the Drosophila optic lobe and the molecular mechanisms of layer specific targeting of R8 axons in the medulla. Fundamental and comprehensive understanding of the crosstalk of growing axons and target regions in the Drosophila optic lobe will elucidate the general principles applicable to more complex nervous systems.

Key words: axon targeting, Drosophila, layer, visual system

INTRODUCTION

During the formation of neuronal circuits, axons find their appropriate targets and form synapses with them to selectively generate a functional network. The fine anatomical architecture of the brain reflects the structured organization of its function. A layered structure is one of the most common patterns seen in the brain of vertebrates and invertebrates. Each layer is composed of particular neuronal functions and connectivity, which facilitates the organization of its input-output relationships. While the layers are compartments along the horizontal axis, neurons are also grouped perpendicularly to the layers, into units called columns. The column is thought to be a functional unit for signal processing tasks in the neuronal network. Neurons in the brains are thus wired vertically and horizontally with astounding precision and fidelity, which enables the network to efficiently process and propagate neuronal information (Huberman et al., 2010).

To achieve this incredibly complex wiring, neurons should extend their axons between the numerous axons and dendrites of other neurons during development, making various decisions such as to go, to turn, to stop, or to defasciculate according to signals from the environment. The visual system of Drosophila has a simplified but similar architecture to that of vertebrates, and has thus contributed to the identification of molecules and signaling mechanisms controlling the wiring specificity of neurons (Sanes and Zipursky, 2010).

In this review, we first survey the development of the layered and columnar structure of the Drosophila visual system, and then discuss the molecular agents involved in the layer-specific targeting of photoreceptor axons. The axonal and dendritic targeting of the Drosophila olfactory system is also discussed in this issue (Sakuma et al., 2014). The hormonal control of neuronal development, as well as recent advances in genome editing in Drosophila, is also reviewed in this issue (Niwa and Niwa, 2014; Kondo, 2014).

DEVELOPMENT OF THE DROSOPHILA VISUAL SYSTEM

The compound eye of an adult Drosophila is composed of approximately 750 ommatidia, and each ommatidium contains eight photoreceptor cells: R1 to R8. The outer photoreceptors R1–R6 express rhodopsin R1 which
responds to a broad spectrum of visible light. The inner photoreceptors R7 and R8 mediate color vision. R7s express the UV-sensitive rhodopsins Rh3 or Rh4, whereas R8s express the rhodopsins Rh5 or Rh6, which respond to blue and green visual inputs, respectively (Wolff and Ready, 1993). Unlike in vertebrates, the eight axons of R1–R8 from a single ommatidium form a single fascicle and project directly into the optic lobe of the brain, which consists of four distinct neuropils: the lamina, medulla, lobula, and lobula plate (Meinertzhagen and Hanson, 1993). During development, the growth cones of R1–R6 axons stop in the lamina, but the growth cones of R7 and R8 axons pass through the lamina and grow into the medulla (Fig. 1A) (Ting et al., 2005).

The lamina is the first optic ganglion organized in radial synaptic units called cartridges. While R7 and R8 lie in the center of the ommatidia, R1–R6 encircle them. Due to the curvature of the eye, R1–R6 photoreceptors from the same ommatidium receive visual input from different angles. Therefore, to maintain retinotopy, one lamina cartridge collects the input from the axons of six photoreceptors whose cell bodies belong to six separate ommatidia, next to each other. To achieve such a connection, a bundle of R1–R6 axons defasciculates to spread laterally into a stereotyped connection pattern in the lamina. This wiring strategy between the retina and lamina is called a neuronal superposition. R1–R6 axons form synapses with a subset of lamina neurons (L1–L3) in a cartridge and transduce the visual information to the second ganglion medulla (Fig. 1B) (Hadjieconomou et al., 2005).

Fig. 1. Schematic drawings of the Drosophila optic lobe. (A) The Drosophila adult retina is composed of approximately 750 ommatidia, each of which contains eight photoreceptor cells, R1 to R8. The axons from the outer photoreceptors R1–R6 (red) innervate the first optic ganglion, the lamina, whereas the inner photoreceptors R7 (green) and R8 (purple) extend into the second optic ganglion, the medulla. The medulla is divided into ten layers, and R7 and R8 target the M6 (pink) and M3 (blue) layers, respectively. The cell bodies of the lamina neurons L1–L5 lie between the retina and the lamina, and their axons extend through the lamina into the medulla together with R7 and R8. (B) R1–R6 projection pattern to a cartridge in the lamina. The R7/R8 axons extend towards the medulla through the lamina cartridge directly below. The axons from the R1–R6 of six surrounding ommatidia, which point to the same direction as R7/R8 of the central ommatidium, converge onto the same cartridge with R7/R8. The photoreceptors focused on the same point in space are colored in each ommatidium. The axons from lamina neurons L1–L5 under the central ommatidium also extend into the same cartridge and R1–R6 axons form synapses with L1–L5 within the cartridge. A schematic of a cross section of the lamina cartridge depicting the photoreceptor axons and lamina neurons is shown aside. (C) The target layers and the corresponding neurons are listed. The R7 and R8 axons from the same ommatidium and axons of lamina neurons L1–L5 from its corresponding cartridges project into a single column in the medulla. They target a distinct layer, and form synapses.
Axon targeting in the \textit{Drosophila} visual system

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2011; Sanes and Zipursky, 2010).

The horizontal organization of R7 and R8 is simpler, since they lie in the center of an ommatidium and are responsive to the same point in space. In the medulla, each functional unit associated with a single R7/R8 projection is called a column. Each column is innervated by 50–60 neurons, including the axons of photoreceptors R7 and R8 and lamina neurons (L1–L5) (Fig. 1C) (Fischbach and Dittrich, 1989). The R7 and R8 axons from the same ommatidium, together with axons of lamina neurons from corresponding cartridges, project into a single column (Mast et al., 2006; Sanes and Zipursky, 2010). The medulla is divided into ten layers M1–M10, to which photoreceptor and lamina neurons target with stereotypic connection patterns (Fig. 1, A and C). R7 and R8 axons terminate in two distinct layers, M6 and M3, respectively (Hadjieconomou et al., 2011; Sanes and Zipursky, 2010).

Together with the powerful genetic tools available, the regular columnar and layered organization of the visual systems of \textit{Drosophila} is well suited for investigating the rules for the development of complex neuronal connectivity. The molecular mechanisms underlying the formation of the \textit{Drosophila} visual system have been widely reported, but here we focus our discussion on the function of the molecules that control the targeting of the R8 axon to the distinct medulla layer M3.

STEPWISE TARGETING OF R7 AND R8

The \textit{Drosophila} compound eye develops from a monolayer epithelium called an eye-disc. The differentiation of eye photoreceptors starts from the posterior tip of the eye-disc in the early third instar larvae and extend anteriorly across the eye-disc as a morphogenic furrow (Wolff and Ready, 1991). Among the eight photoreceptors in an ommatidium, R8 is the first cell to be specified, inducing the differentiation of the remaining R2/R5 and R3/R4, then R1/R6, and finally R7. The pioneer R8 axons grow towards the optic lobe through a narrow tube called the optic stalk, which connects the eye-disc and the brain. After entering the brain, axons spread, maintaining their positions according to the retina, and thus forming a topographic map. The R1–R7 axons from the same ommatidium follow the pioneer R8 axon trajectory until they reach the lamina. R8 axons, and later R7 axons, go through the lamina and extend towards the developing medulla (Fig. 2A) (Taylor and Garrity, 2003). Although R7 and R8 photoreceptors position themselves in the center of the ommatidium and their axons grow out from the same point of the retina, their behavior towards the specific layers in the medulla is different. After reaching the medulla, R8 axons pause at the R8 temporary layer that lies at the apical surface of the medulla neuropil. The R7 axons following them overtake the pausing R8 and enter the medulla to the deeper R7 temporary layer.

As the R8 and R7 start extending axons to the medulla in temporal order, the gradient of the R7 axon terminals according to their time of birth can be observed at this stage. Meanwhile, lamina neurons also grow and establish stereotyped arborizations in medulla layers, which increases the distance between the R7 and R8 temporary layers. The second step of medulla targeting occurs after all the axons of R7, R8, and lamina neurons reach their respective temporal layers, when R8 axons start to extend thin filopodia towards their final targets at around 50 hr APF (after puparium formation) (Fig. 2B). By 70 hr APF, R7 and R8 axons reach their final target layer, develop mature terminals, and undergo synaptogenesis. Two important steps for correct R8 targeting are when R8 stays at the R8 temporary layer at the surface of medulla, and the stage when R8 begins to extend its axons again from the R8 temporary layer and reaches the final layer. Several key molecules contribute to regulating each step to perform R8-specific axonal growth according to the optic lobe developmental stage (Fig. 2C) (Hadjieconomou et al., 2011; Ting et al., 2005).

GOLDEN GOAL AND FLAMINGO

An interaction between two cell surface molecules, Golden goal (Gogo) and Flamingo (Fmi, also known as Starry night, Stan), is the key to guiding R8 axons to their proper target layer. Fmi is a seven-pass transmembrane cadherin which has multiple functions (Berger-Muller and Suzuki, 2011). It is required in photoreceptor targeting (Lee et al., 2003), in dendritic formation (Gao et al., 2000; Kimura et al., 2006), and in planar cell polarity (PCP) (Chae et al., 1999; Usui et al., 1999). Gogo is a putative receptor which is also involved in photoreceptor targeting and dendritic formation (Berger et al., 2008; Hakeda-Suzuki et al., 2011; Hakeda and Suzuki, 2013; Heintz et al., 2013; Mann et al., 2012; Ohler et al., 2011; Tomasi et al., 2008). Both Gogo and Fmi are expressed in photoreceptor axons in the third instar larvae, and are strongest in the youngest axons. In the optic lobe, Fmi is strongly expressed in the target region together with photoreceptor axons, whereas Gogo is mainly detectable in photoreceptor axons. \textit{gogo} mutants have a very similar phenotype to \textit{fmi} mutants in photoreceptor axon guidance (Hakeda-Suzuki et al., 2011; Lee et al., 2003; Sentii et al., 2003; Tomasi et al., 2008). In the medulla of eye-specific mutants of \textit{gogo} and \textit{fmi}, the majority of R8 photoreceptor axons fail to send filopodia toward the M3 target layer at 50 hr APF when R8 axons restart their growth. As a consequence, many R8 axons stay at the R8 temporary layer and fail to innervate the medulla. In spite of the similarities in the mutant phenotypes, Gogo and Fmi play distinct roles in R8 targeting; the functional association of these two molecules is only temporarily achieved. Gogo is solely required in the first step when
R8 must adhere to the R8 temporary layer, whereas the functional association between Gogo and Fmi occurs from the mid-pupal stage onwards. After Fmi inhibits the Gogo interaction with the temporary layer, Gogo and Fmi act together to promote R8 axons to the M3 layer. Fmi expression in target cells is necessary, but the Fmi cytoplasmic domain is dispensable in R8 targeting, suggesting that Fmi mediates the axon-target interaction with the cell adhesion property of its extracellular domain. On the contrary, Gogo expression in the target cells is not
involved in R8 targeting, but the Gogo cytoplasmic domain is necessary, indicating that Gogo mediates intracellular signaling to transduce axon pathfinding information into the growth cone (Hakeda-Suzuki et al., 2011).

The Gogo protein has a conserved YYD tripeptide motif in the cytoplasmic domain and the phosphorylation status of its tyrosine is critical for its collaboration with Fmi to guide R8 axons for properly targeting the M3 layer. Phosphorylated Gogo enhances the adhesive interaction of R8 with the R8 temporary layer, whereas dephosphorylation provides a permissive signal that allows the axon to leave the temporary layer and project to the M3 targeting layer (Mann et al., 2012). Although phospho-mimicking Gogo enhances the adhesiveness of the R8 axon to the R8 temporary layer, and Gogo is indeed phosphorylated at an early stage of pupal development, the biological relevance of the phosphorylation has to be further examined, as dephosphorylated Gogo can apparently rescue the R8 axon targeting of gogo mutant flies.

**NETRIN AND FRAZZLED**

Netrins (Net) are secreted chemotropic guidance molecules that were identified as a bifunctional guidance cue for growing axons (Harris et al., 1996; Ishii et al., 1992; Kennedy et al., 1994; Lai Wing Sun et al., 2011; Mitchell et al., 1996; Serafini et al., 1994, 1996). The attraction by Net is mediated by the receptor Frazzled (Fra), the *Drosophila* homolog of Caenorhabditis elegans UNC-40, whereas the receptor UNC-5 mediates repulsion (Hong et al., 1999; Keleman and Dickson, 2001; Kolodziej et al., 1996; Leonardo et al., 1997; Leung-Hagestijn et al., 1992; Round and Stein, 2007).

*Drosophila* has two Netrins, Netrin-A (NetA) and Netrin-B (NetB). Both Netrins are expressed in a single medulla layer M3, while only the expression pattern of NetB is examined in more detail (Timofeev et al., 2012). In the developing medulla, NetB can be detected in the emerging M3 layer at 42 hr APF. Its expression gets strongest at 55 hr APF. The receptor Fra is expressed in R8 axons as well as in target neurons at the M3 layer at 42 hr and 55 hr APF. R8 axons which lack Fra expression reach the R8 temporary layer and pause there correctly, but fail to extend further at the second step and remain at the medulla surface or target between M3 and M1 in the adult. Consistent with the attractive function of Fra, the R8 axons which innervate the optic lobe that lacks both Netrin isoforms (NetA and NetB) in *Drosophila*, show premature innervation to the M1 or M2 layers. The expression of NetB in L3 lamina neurons, which target the M3 layer in the Netrin-deficient brain, can direct the R8 axons to the proper M3 layer. Furthermore, high levels of NetB in the M1/M2 layers can cause some R8 axons to stop at a shallower layer, despite endogenous Netrin expression at the M3 layer (Timofeev et al., 2012). These findings suggest that the Net/Fra system plays an instructive role in attracting R8 axons, and leading them to their proper layer. Fra expressed in target neurons captures the Netrin to restrict the Netrin expressing region as in the embryonic CNS (Hiramoto et al., 2000; Timofeev et al., 2012). The Netrin protein level is clearly decreased in the medulla region that lacks Fra, however, the requirement for Fra in the target region for R8 axon targeting remains to be clarified.

**CAPRICIOUS**

Capricious (Caps) is a transmembrane protein with leucine-rich repeats. It was originally found to play a role in synapse formation at the neuromuscular junction in *Drosophila* larvae. Caps is expressed in a specific and paired pattern between a subset of motoneurons and the muscles that they innervate, thus mediating axon target recognition with its homophilic cell adhesion property (Abrell and Jackle, 2001; Kohsaka and Nose, 2009; Shishido et al., 1998; Taniguchi et al., 2000). In the developing visual system, Caps shows specific expression in R8 axons and several medulla layers, including the R8-receptor layer M3, leading to the idea that Caps mediates R8-target recognition in a similar manner as it does to motoneurons. An initial report shows that R8 axons that lack Caps abnormally terminate in a more superficial or deeper layer than the M3, and some extend into neighboring columns. It has also been shown that overexpression of Caps in R7 causes the mistargeting of R7 axons from the correct layer (M6) to the R8-receptor M3 layer (Shinza-Kameda et al., 2006). Contrary to this report, another recent study shows that mistargeting of Caps-overexpressing R7 to the M3 layer does not require Caps expression in the target region. Moreover, caps mutant photoreceptors showed a very mild overshooting phenotype that is much weaker than that described in the initial report. These findings indicate that Caps does not mediate the R8 photoreceptor targeting by R8-target adhesion (Berger-Muller et al., 2013). However, Caps specific expression and its adhesive property still imply its function in R8 targeting, and the discovery of additional molecules such as a functionally redundant protein, or a heterophilic ligand, is expected.

**CONCLUSION AND PERSPECTIVES**

Neuropils are composed of axons, dendrites, and glial cell processes. Each protrusion has to make its own decisions on how to behave according to the surrounding information. In the *Drosophila* medulla, various types of axons innervate the same column, but they must target distinct layers, receiving different commands on their way to those layers although they are proceeding on the same tract. Photoreceptor R8 axons of *Drosophila* take
characteristic steps to extend toward their target layer. They grow, pause, grow again, and stop. On the contrary, R7 axons keep growing until they reach their final targets. Thus, the targeting of R7 and R8 photoreceptors serves as a good model to analyze the mechanisms underlying layer specific targeting.

Two key interactions are reported to contribute to the second step of R8 axon targeting when they regrow: Gogo/Fmi and Net/Fra. In both combinations, the molecules from the targeting cells induce a cell-cell attraction to the R8 axons, and lead to the proper layer. While both pathways are genetically required for proper R8 axon targeting, the degree of sufficiency differs. Gogo/Fmi is sufficient to retarget R7 axons to the R8 recipient M3 layer, while Net/Fra is not. The expression pattern of Net/Fra shows more specificity than Gogo/Fmi; Netrin is largely M3 specific, which attracts attention, while Fmi and Gogo are expressed both in R7 and R8, with broad expression pattern in multiple medulla layers. To explain R8 targeting with the Gogo/Fmi pathway, an additional piece of evidence is required: why Fmi expressing R8 axons ignore the Fmi expression in other layers such as M1 and M5, but specifically and homophilically recognizes the M3 layer. A fascinating hypothesis is that Netrin is the M3 specific cue to strengthen the Fmi homophilic interaction between R8 axons and their targets. However, there are several observations that speak against this hypothesis. First of all, net mutants have a much milder phenotype than fmi mutants. Second, the identical phenotypes of net and fra mutants do not allow us to predict any more intercessory molecules. Although Gogo/Fmi and Net/Fra seem to be independent, there may yet be new evidence to explain the functional interaction between these two pathways.

Gogo is the only molecule known to affect the first step of R8 growth, i.e. to cause the axons to stop at the R8 temporary layer in the early pupa for 48 hr. However, even in gogo mutants, only a few R8 axons pass through the R8 temporary layer and mistarget to the deeper layer. Still a critical cue is missing for the first step of R8 temporary layer and mistarget to the deeper layer. A fascinating hypothesis is that Netrin is the M3 specific cue to strengthen the Fmi homophilic interaction between R8 axons and their targets. This was supported by Grant-in-Aid for JSPS Fellows (S. H-S), Grant-In-Aid for Research Activity Start-up (24800024), and Grant-In-Aid for Scientific Research on Innovative Areas (25110713), Tomizawa Jun-ichi & Keiko Fund of Molecular Biology Society of Japan for Young Scientist, Mitsubishi Foundation, Takeda Science Foundation, Sumitomo Foundation, Life Science Foundation, Itoh Foundation, Inamori Foundation, Daiichi-Sankyo Foundation and Toray Science Foundation (T.S.).

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