Temporal and spatial order of photoreceptor and glia projections into optic lobe in *Drosophila*

Yen-Ching Chang\(^1,2\), Chia-Kang Tsao\(^1,2\) & Y. Henry Sun\(^1,2\)

Photoreceptor (PR) axons project from the retina to the optic lobe in brain and form a precise retinotopic map in the *Drosophila* visual system. Yet the role of retinal basal glia in the retinotopic map formation is not previously known. We examined the formation of the retinotopic map by marking single PR pairs and following their axonal projections. In addition to confirming previous studies that the spatial information is preserved from the retina to the optic stalk and then to the optic lamina, we found that the young PR R3/4 axons transiently overshoot and then retract to their final destination, the lamina plexus. We then examined the process of wrapping glia (WG) membrane extension in the eye disc and showed that the WG membrane extensions also follow the retinotopic map. We show that the WG is important for the proper spatial distribution of PR axons in the optic stalk and lamina, suggesting an active role of wrapping glia in the retinotopic map formation.

The visual system of both vertebrates and invertebrates consists of light-sensing photoreceptor (PR) neurons in the retina connected to the inner layers of neurons to form a precise retinotopic map for visual information processing\(^1\)–\(^3\). In the vertebrate retina, the PR axons project inward to form synapses with bipolar cells at the outer plexiform layer. The bipolar cells then project inward to make synaptic connections with the retinal ganglion cells (RGC) at the inner plexiform layer. Within each layer, the neurons are connected by interneurons for information integration. The RGCs axons then exit the retina and make connections with the optic tectum or lateral geniculate nucleus (LGN) in the brain in a spatially precise one-to-one retinotopic map. The retinotopic map preserves the spatial information detected by the PR and transmit into the brain for further information processing\(^1\)–\(^3\). The formation of the retinotopic map is dependent on gradients of guidance molecules and on activity-dependent interactions among axons\(^1\)–\(^4\).

The highly regular and repeated structure of the *Drosophila* visual system makes it an excellent experimental model to study mechanisms regulating the formation of the retinotopic map. The *Drosophila* compound eye consists of around 800 ommatidia, each with eight photoreceptors (R1-8) plus a number of accessory cells\(^5\). The PR axons project into the optic lobe in a spatially precise one-to-one retinotopic map, i.e. maintaining their relative anterioposterior (A-P) and dorsoventral (D-V) order (Fig. 1A). The spatial precision of the PR axonal projections into the optic lobe has been examined in detail in the adult brain of larger insects and of *Drosophila*\(^6\)–\(^11\). The fly eye also provides a great opportunity to study the developmental progression of axonal projections and the formation of the retinotopic map (reviewed by\(^12\)). The fly compound eye develops from the larval eye imaginal disc, which is connected to the brain via the optic stalk (OS). The eye disc is a single epithelial cell layer, covered with a single apical peripodial membrane that does not contribute to the adult eye. During the third instar larval stage, the eye disc begins to differentiate in a progressive wave moving from the posterior end to the anterior portion. Cells at the front of the wave transiently shorten and form a morphogenetic furrow (MF) along the D-V direction. As the D-V-oriented MF moves anteriorly, cells behind the MF begin to progressively differentiate into rows of ommatidial clusters consisting of photoreceptors\(^5,13\). The PRs differentiate in the sequence of R8 - R2/5 - R3/4 - R1/6 - R7\(^13\). The newly differentiated PRs extend axons basally and then posteriorly along the basal surface, go through the OS and into the optic lobe. The R1–6 axons terminate in the optic lamina and the R7 and R8 axons extend further and enter the medulla\(^14\). The R1–6 growth cones terminate between the rows of epithelial glia and marginal glia, forming the lamina plexus\(^15\). During the pupal stage, the R1–6 axons defasciculate to undergo extensive rearrangement and make synapses with lamina neurons. The R7 and R8 axons terminate at different layers in the

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\(^1\)Institute of Genomic Sciences, National Yang Ming University, Taipei, Taiwan. \(^2\)Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan. Correspondence and requests for materials should be addressed to Y.H.S. (email: mbyhsun@gate.sinica.edu.tw)
plexus36–38. In the eye disc, a group of glia, the retinal basal glia (RBG), migrates from the OS into the basal layer.

Figure 1. Retinotopic mapping of PR axonal projections in OS and optic lamina. Kaede expression in R3/4 is driven by mβ0.5-GAL4. (A) Schematic drawing summarizes the axon projections of single R3/4 pairs from different A-P and D-V positions in the eye disc into the OS and lamina. Samples were either live discs mounted in low gelling agarose (B–G) or fixed discs mounted on slides (H–J) (N = 7 for live discs and N = 10 for fixed discs). At least 4 single R3/4 pairs were analyzed for each disc. All respected their relative A-P and D-V order. The fixed samples provided better resolution in lamina. Axon projections were traced through different Z-sections. (H–J) Two copies of mβ0.5-GAL4 were used. (B–D),(E–G) and (H–J) are three disc samples. (B,E) Z-projections of XY plane are shown to indicate four photoactivated positions (numbered 1–4) in each disc. (C,F) In the OS, the older axons (#3 and #4) are found in the apical region. In both samples, the most anterior #1 axon has not reached OS and lamina. The anterior-dorsal #2 axon (B) projects to basal-dorsal position (C). The anterior-ventral #2 axon (D) projects to basal-ventral position (E). (D,G) The labeled axon projections (red) in the lamina were 3D-reconstructed by IMARIS and merged with all R3/4 axons (cyan). These axons maintain their relative D-V and A-P positions as in retina. (H) Z-projection shows the position of three photo-activated R3/4 pairs (#1–3) and their axon projections in the lamina, with a higher magnification view of the lamina in the same sample from a different angle (H'). The 3D reconstruction is viewed in two optical cross sections at the red dashed line (I) and white dashed line (J') in H'. (I) The #1–3 axons maintained their relative D-V and A-P positions. (J') In this lateral view of the lamina, the #3 (posterior, older) axon terminate at the lamina plexus (white arrowhead), and the #1 (anterior, young) axon overshoot the lamina plexus. Cyan (green Kaede)/red (red Kaede) presentation is for sample in agarose. Green (green Kaede)/magenta (red Kaede) presentation is for fixed samples. Scale bars are 20 μm for BEH and 10 μm for CDFGH'IJJ'.

medulla and form synapses with lamina neurons16–18. In this study, we will focus on the formation of the first layer of the retinotopic map, i.e. the projection of PR axons to the lamina during the larval stage.

The overall organization and design principle of the *Drosophila* retina/ lamina/medulla and the vertebrate retina/inner and outer plexiform layers is very similar19–21. Therefore, the study of fly retinotopy can provide useful insight for the understanding of the development of the mammalian visual system. Earlier studies have examined the retinotopic projections of PR axons by electron microscopy (EM) analysis in larger insects8–10. The retinotopic map in *Drosophila* was examined by using the lipophilic dyes DiI and DiO to label the dorsal and ventral PR, respectively, the *omb*-lacZ to mark the dorsal- and ventral-most PR axons, and the temporal difference in expression timing of anti-horseradish peroxidase (HRP) and monoclonal antibody 24B10 staining to distinguish the younger and older axons, and followed their axonal projections into OS and optic lobe22,23. The HRP is a neuronal marker24 that stains the core α1–3-fucosylated N-glycan on neuronal cell surface proteins25,26. The 24B10 staining specifically PR cell body and axon27 by recognizing the cell surface glycoprotein Chaoptin28,29. Because the HRP signal appears in differentiating PR about 9 hr. (at 25 °C) before the 24B10 signal, the most anterior (youngest) six or seven rows of ommatidia are labeled by anti-HRP but not by 24B1027. Thus HRP and 24B10 double staining can be used to distinguish the younger and older axons30. Marked PR clones were also analyzed30. The results showed that the PR axons follow the A-P (younger-older) and D-V order in their projection into the OS and optic lamina. However, because, a group of axons were labelled in these experiments, the spatial resolution of PR axon development is limited. In this study, we used the photoconvertible fluorescent protein Kaede31–33 to explore this question. UV irradiation induces a peptide cleavage of Kaede proteins, which results in the rapid and irreversible conversion from green fluorescence to red fluorescence34,35. By expressing Kaede in specific PRs and selectively photo-activating PR at specific positions, we can follow PR axonal projections into OS and lamina with single axonal pair resolution.

The vertebrate retinal neurons are ensheathed by Müller glia and astrocytes (reviewed by34,35 but the role of these glia in the formation of retinotopic map has not been reported. Glia is involved in the PR axonal projection in *Drosophila*. In the optic lamina, glia is required for the proper termination of R1–6 axons in the lamina plexus36–38. In the eye disc, a group of glia, the retinal basal glia (RBG), migrates from the OS into the basal layer of the eye disc39,40. Their migration into the eye disc follows the PR differentiation and they always lag behind the
Young R3/4 axons transiently overshoot in lamina. To achieve better spatial resolution of PR axons in the lamina, we used another method to analyze the projection pattern of young versus older PR axons. PR differentiate in the order of R8 = >R2/5/7 = >R3/4/6 = >R1/6/7. We labeled R3/4 and R7 by expressing mCD8GFP and Kaede driven by R3/4-specific m0.5-GAL4 and R7-specific sec181-GALA, respectively. Since R3/4 differentiate earlier than R7, there is an anterior region (6-7 rows of ommatidia) with only R3/4 expressions, allowing specific photoconversion of the young R3/4 PRs. The anterior (younger) R3/4 are labelled by red Kaede and green GFP, while the more posterior (older) R3/4 are labelled by both green GFP and green Kaede, so the differential red-to-green ratio allows easy distinction of young vs older R3/4 axons (Fig. 3A). From the lateral view of eye disc, it is clear that the younger R3/4 axons lay at the basal layer, while the older R3/4/7 axons are located in more apical position (Fig. 3C-C), consistent with our previous findings (Figs 1 and 2). In the lamina, the young R3/4 axons project to the anterior position (Fig. 3E).

Interestingly, we found that whereas the older R3/4/7 axons (green) terminate in a neat line (termed the lamina plexus) in the lamina (Fig. 3E) as previously reported for all R1-6 axons55, the younger R3/4/7 axons (red) terminate in deeper positions in the lamina, and even in the medulla (Fig. 3D,F,F'). Some overshooting can also be observed in m0.5 > Kaede (Fig. 1; Supplementary Fig. 2). m0.5 > mCD8GFP also showed similar phenomenon (Supplementary Fig. 4B). We generated MultiColor FlpOut (MCFO) clones48 driven by the m0.5-GALA. In these single cell clones, the younger R3/4/7 axons also showed overshooting, but the older R3/4/7 axons terminate at the lamina plexus (Supplementary Fig. 4C). These results suggest that the young R3/4 axons transiently overshoot and then retract back to assume their final destination in lamina plexus.

We tried to ask whether this transient overshoot of R2/5 axons is a general property of all R1-6 axons. To see this early event, we need early markers for photoreceptors. The rhodopsins express only in well differentiated PR, so is not suitable for this purpose. The 24B10 stains all PR axons, but does not show any overshoot in lamina (e.g. Yu et al.50), presumably because its expression is later than the early event. Ro-tau-lacZ marks R2-5 axons51 and showed some axonal overshoot52. However, since its expression includes R3/4, whether R2 and R5 axons also overshoot is not clear. MT14-GALA4 is reported to be specific for R2/5/856, although also reported to have some expression in other PRs.56 We found that MT14 > mRFP axons do not show overshooting in lamina (Supplementary Fig. 4D). We generated MCFO clones driven by the MT14-GALA. In these single cell MCFO clones, the younger PR and older PR all have axons terminate at the lamina plexus (Supplementary Fig. 4E). However, all PR cells labelled by MT14 > mRFP (magenta) are also 24B10', therefore the MT14-GALA expression only marks older R2/5/8 axons. Therefore, whether the young R2/5/8 axons also overshoot cannot be determined using this reporter.
WG membrane extension into optic lamina. We examined the axonal ensheathment process at single glia cell resolution by using hs-FLP and repo-GAL4 to generate flip-out clones\(^\text{57,58}\) and monitored these clones by live imaging of ex vivo cultured eye disc (Supplementary Fig. 5). The glial membrane is visualized by mCD8-GFP. The WG and SG can be distinguished by their distinct morphologies. The SG can undergo cell division (Supplementary Fig. 5, yellow arrowheads). The WG progressively extends its membrane posteriorly toward the OS, presumably along the wrapped axons, with its nucleus move up and down in the cell (Supplementary Movie 1; Supplementary Fig. 5, blue and red arrowheads). We also used transmission electromicroscopy (TEM) to examine the axonal ensheathment by WG membrane. CD2-HRP\(^\text{59}\) was driven by the WG-specific MZ97-GAL4 and stained by Diaminobenzidine (DAB) to detect WG membrane. In a cross section of eye disc, WG membrane can be found infiltrating to PR layer and membrane surround the PR axon (Supplementary Fig. 7A1). The WG membrane can also be noted extending into the lamina (Supplementary Fig. 7AII).

We then examined the temporal progression and the extent of WG membrane extension. Although Mz97-GAL4 is expressed only in WG in eye discs\(^\text{44}\), it also expressed in some glia in the optic lobe (data not shown). Therefore, this GAL4 is not suitable for tracing retinal WG membrane extension into the optic lobe. To overcome this issue, we screened the Janelia Farm collection of GAL4 lines with retinal glia expressions and identified a GMR74E02-GAL4 (referred to as WG-GAL4 in this study) that marks only the retinal WG but no other glia in the optic lobe and brain (Supplementary Fig. 6A,B). Examination of eye discs of different developmental age showed that the progressive WG membrane extension lags behind the PR axon projection through OS to lamina (Supplementary Fig. 6D–F). The WG membrane extends into lamina but stops at the anterior layer of lamina. When all glial membrane is labelled, we noticed a region in lamina that lacks glial membrane (Supplementary Fig. 7AII).
The termination of WG membrane extension is not because of touching the next lamina glial membrane. Since PR axons continue to extend into the lamina (R1-6) and medulla (R7/8), there is a segment of PR axon not wrapped by glial membrane. We also generated WG MCFO clones using the WG-GAL4. The WG membrane extension is not because of touching the next lamina glial membrane. Since PR axons continue to extend into the lamina (R1-6) and medulla (R7/8), there is a segment of PR axon not wrapped by glial membrane. We also generated WG MCFO clones using the WG-GAL4. The WG membrane.

Figure 3. Young R3/4 axons transiently overshoot in lamina. mCD8GFP and Kaede expression are simultaneously driven by R3/4-specific mδ0.5-GAL4 and R7-specific sev^{181}-GAL4. Anterior is to the left for all images except (E). (A) HRP staining (cyan) shows all PR cell soma and axons. Young R3/4 cells are labeled by red Kaede (magenta) and mCD8GFP (green); mature R3/4/7 PRs are labeled by green Kaede and mCD8GFP. Box indicates the region of UV illumination to convert Kaede. (B) R3/4 can be distinguished by cell shape. (C′-C″) Lateral view shows that HRP (cyan) appear before R3/4 axonal projections (red), which is anterior to R3/4/7 positive region (green). The extents of the three color signals are shown below. mδ0.5 > Kaede+ mCD8-GFP has low level of GFP in R3/R4 that is not apparent and is not shown in the colored bars. The young R3/4 axons (red) are basal to the older R3/4/7 (green) axons. (D) Young R3/4 axons (magenta) project past the lamina plexus while the older R3/4/7 (green) terminate at the lamina plexus in the lamina. The overshoot R3/4 axons costains with HRP (blue) (D′), indicating that these are axons. (E,F) The projections can be viewed in two optical sections (red and white dashed lines in D′) in 3D reconstruction. (E,E′) The young R3/4 axons (magenta) terminate in the anterior portion of lamina, while the older axons (green) terminate in more posterior regions. (F-F″) Lateral view of lamina shows that the overshoot young R3/4 axons (arrowhead) is at the anterior portion and past the lamina plexus (arrow) marked by the older PR axon termini. The brackets indicate the lamina and medulla regions. The asterisk indicates the background of Kaede. (H) Schematic drawing of eye-brain complex showing rows of R3/4 and R3/4/7 in a late third instar eye disc with ~20 rows of ommatidia. The young R3/4 axons (anterior, magenta) project past the lamina plexus, while the older R3/4/7 axons (posterior, green) terminate at the lamina plexus. Scale bar is 5 μm for (B), 10 μm for F-F″, 15 μm for C-C″, 20 μm for A and E-E′ and 30 μm for (F).
extends to the lamina neurons L1-L4, but not to the lamina L5 neurons and the lamina plexus (Supplementary Fig. 7B). The younger WG membrane extends to adjacent to the lamina neurons, while the older WG membrane extend to partially surround the lamina neurons (Supplementary Fig. 7C). Consistently, recent reports showed that WG membrane progressively extend into the lamina36,47.

**WG membrane extension also follows the retinotopic order.** In order to clearly demonstrate the A-P and D-V relationship of retinal WG and their membrane extension into lamina, we used two methods to clonally label WG cells. Both methods are based on the flip-out concept42. A transient heat shock during development induces the expression of hs-Flip to randomly induce the excision (flip-out) of a stop cassette in UA5-reporters in cells. The UA5-reporter is then responsive to GAL4 induction. In the first flip-out method, the UA5 > CD2, y+ > mCD8GFP57,58 and Mz97-GAL4 were used. The hs-Flip was induced transiently 12 hr. before late third instar larva. If the flip-out cell and its progeny cells differentiate into WG, then the Mz97-GAL4 will drive the expression of mCD8GFP. We found that 88% of WG clones (N = 42) has only single cell, suggesting that during the 12 hr. since heat shock induction, the flip-out cell rarely underwent cell division. The advantage of this method is that live imaging is possible, but the distinction of neighboring clones is not easy. Multiple clones in a disc are numbered and the extent of their membrane, marked by mCD8GFP, can be traced. Because we never observe WG membrane extend anteriorly (Supplementary Fig. 4), the anterior extent of its membrane is interpreted as an indicator of its time and position of origin (differentiation into WG), i.e. the posterior #2 WG is older than the anterior #1 WG (Supplementary Fig. 8A). Like the PR axons, the younger WG (#1) membrane -presumably wrapping younger PR axons- is located in a more basal position in the OS than the position of older WG (#2) (Supplementary Fig. 8C,D-D’). The lateral clones #3 and #4 maintain their relative D-V positions in the OS and the medial #1 and #2 maintain their medial position (Supplementary Fig. 8C).

The second method uses the MCF049 and the WG-GAL4 to label multiple WG clones with different combinations of the V5, HA, FLAG tags on myristoylated (membrane) GFP, as such the extent of membrane extension can be observed. This method provides better distinction of neighboring clones. The heat shock induction was 24 hr. prior to late third instar larva, therefore most clones contain multiple cells. Similar results were obtained with Mz97-GAL4 driven MCF0 analysis. WG clones preserve their relative D-V positions (Fig. 4 and Supplementary Fig. 9). In Fig. 4B, 5 WG clones were labelled: anterior WG clones (AV1, AV2) and posterior WG clones (PD1, PD2 and M). Membranes from anterior clones (younger WG) are located in the basal portion of OS, compared to those from posterior WG clones (Fig. 4C,D). The WG membrane extension follows the retinotopic rules of PRs in maintaining their D-V orders when projecting into optic lobe (Fig. 4E–H). Similar to the PR axons, the younger WG membrane extension stops at a more anterior position in the OL, while the older ones are at more posterior positions.

**WG affects PR axon projection in OS and lamina.** Since the wrapping of axons is temporally tightly coupled with the extension of PR axons into the OS and lamina, we tested whether the wrapping affects the axonal retinotopic projection. In the OS, the central region consists of axons wrapped by WG membrane41, visualized by WG > mCD8-GFP (Fig. 5E,F). The periphery of OS consists of SG membrane41. FGF signaling pathway has been reported as both necessary and sufficient to promote WG differentiation43. In pan-glial knock down of the FGF receptor Heartless (Htl), the WG does not differentiate and there is no WG membrane in the center of the OS (Fig. 5D, compare with 5B), indicating the loss of WG membrane. The periphery of OS consists of SG membrane, so is not affected by Htl knockdown. When Htl is knocked down specifically in differentiated WG by the WG-GAL4, the WG membrane in OS is not obviously affected (Fig. 5H), although previous study showed that membrane extension into lamina is affected47.

The young and old PR axons are distinguished by the relative timing of 24B10 and HRP staining42. HRP-only axons (cyan) represent younger axons, while 24B10-positive axons (magenta) represent older axons (Fig. 5). In wild type, about 25% (N = 6 for repo-Gal4 control and N = 17 for WG-Gal4 control) of the 24B10-positive axons are in the basal volume. The percentage increased to 39% in pan-glial Htl knockdown (N = 8) and 38% in WG-specific Htl knockdown (N = 11) (Fig. 5L,J). The 24B10-+ axons in the Htl knockdown OS also appears to be more dispersed than in controls (Fig. 5L,J). In mutant animals, axons layers become extremely flattened. In addition, young (HRP staining only) and old (24B10) axons were patchily distributed. Additionally, WG membrane extends into the OS and lamina, but terminate in lamina in an irregular manner (Figs 5F,H and 6D,C). Similar defects of membrane extension were previously reported while overexpressed Hid driven by Mz97-GAL447,48. In the lamina, when dominant-negative Htl was expressed in WG, the lamina plexus is less compact than in wild type (Fig. 6C,D, compare with 6A,B). A projection from a different angle shows that the WG > Hid47 lamina has some gaps in the lamina plexus (Fig. 6C, compare with Fig. 6A). These results showed that the reduction of Htl in WG affects PR axon retinotopic projections in OS and lamina.

We examined the effect of WG on PR retinotopic projection by other manipulations. We first killed WG by expressing the pro-apoptotic genes head involution defective (hid) and reaper (rpr) using WG-GAL4. Since the continuous expression of death genes in WG > hid + rpr lead to larval death, we used tub-GAL80o to temporally control the death genes expression (abbreviated as WGo > hid + rpr). After a 12 hr. induction of hid and rpr expression in late third instar larvae, there is significant increase of the apoptotic marker cleaved caspase 3 (Fig. 7A,E) and significant decrease of Cut+ cells in the eye disc (Fig. 7B,F), indicating the killing of a significant proportion of WG cells. The preference of younger PR axons to occupy the more apical region in OS is reduced (Fig. 7C and G). In the lamina, there is more and larger holes (Fig. 7D,H), suggesting a more disorganized retinotopic distribution. These results demonstrate that WG is important for the proper retinotopic projection of PR axons. When CG is similarly killed (in C135o > mCD8GFP + hid + rpr), the number of WG is slightly reduced, the PR retinotopic projection pattern in OS is not affected, but there are more holes in the lamina (Supplementary Fig. 10G–I). When hid were driven by the SG-specific CS27-GAL4, the number of WG was not affected. Although
WG is derived from SG, the 12 hr of hid induction was too short to significantly affect the transition from SG to WG. In C527ts > hid, the PR retinotopic projection pattern in OS and in lamina were not significantly affected (Supplementary Fig. 10O-R). These results showed that the WG has a major role in PR retinotopic projection in OS and in lamina.

We also blocked WG membrane extension to test for the effect on PR retinotopic projection. The Borderless (Bdl) receptor is expressed in WG to receive the signal Turtle (Tutl) expressed in the PR axon. We knocked down Bdl in WG (WG > mCD8GFP + Bdl-RNAi). As previously reported, Bdl knockdown reduced WG membrane extension in the lamina (Supplementary Fig. 11D-D'). The retinotopic projection pattern in OS is not affected (Supplementary Fig. 11F). The AD clone occupies a basal position in the OS and has not yet reached the lamina. The AM clone occupy a basal position in OS, and has just entered the lamina. The PM clone occupies an apical position in OS and terminated in a posterior position in lamina. Dashed line outlines the OS cross section (C,F).

( Supplementary Fig. 11E-H) shows the locations of PM and AM membrane in lamina. Scale bars are 30 μm for B, 10 μm for CEG, and 5 μm for (F).

Figure 4. WG membrane extension follows the retinotopic rules as PR axons. Multicolor FlipOut (MCFO) clones were generated using WG-specific GAL4 (abbreviated WG > MCFO). Anti-V5 (magenta), anti-HA (green) and anti-Flag (blue) antibodies are used to visualize membrane of WG clones. Clones are named by their relative positions along the A/P and D/V axes of the eye disc. (A) Schematic drawing shows the types of WG clones (white). Green indicates the extent of all WG membrane from eye disc (left) to the anterior edge of lamina (right). (B,E) WG > MCFO clones in eye discs are shown with their projections into the lamina. Dorsal is up and anterior is to left. Several WG > MCFO clones can be identified by different colors due to different reporter combinations. (C) An optical cross-section of the sample in (B) at the OS. The two anterior-ventral WG clones (AV1, AV2) show WG membrane in the basal-ventral region in OS. The two posterior-dorsal clones (PD1, PD2) have membrane in the more apical-dorsal position in OS. The medial (M) clone occupies a medial position in both A-P and D-V axes in the eye disc and in the OS. (D) Schematic summary of the spatial distribution of the clones in (B-C). (E) Among the WG > MCFO clones, an anterior-dorsal (AD), an anterior-medial (AM) and a posterior-medial (PM) clone are labeled and their membrane projections traced into the OS (F) and lamina (G). The AD clone occupies a basal position in the OS and has not yet reached the lamina. The AM clone occupy a basal position in OS, and has just entered the lamina. The PM clone occupies an apical position in OS and terminated in a posterior position in lamina. Dashed line outlines the OS cross section (C,F).

Discussions

Retinotopic map preserves the spatial information from retina to OS and lamina. In this study, we focus on the retinotopic map from retina to lamina. Our single-cell analyses of PR showed that the relative A-P and D-V positions are preserved from the retina to the OS and to the lamina, confirming previous findings. The D-V axon guidance information is provided by the gradient of the DWnt4 expressed in ventral lamina and the receptor Dfrizzled2 along with its downstream signaling component disheveled in the retinal axons, respectively. The dorsal-specific transcription factor Iroquois acts in dorsal PRs to attenuate the response to DWnt4. However, lamina DWnt4 gradient is unlikely to act in the OS. Therefore, the maintenance of the retinotopic map in the OS may rely on as yet unidentified guidance system or on the interaction among neighboring axons.
Transient overshoot of young R3/4 axons in lamina. R1-6 PR axons terminate in the lamina plexus between the layers of epithelial glia and marginal glia. Such termination of R1-6 axons in lamina is dependent on the interaction between the PR axons and lamina glia. Unexpectedly, we found that the young R3/4 axons overshoot and pass the lamina plexus to medulla (Fig. 3). This is not observed in older R3/4 axons, suggesting that the overshoot is a transient event (Fig. 8B). This transient axonal overshoot has not been reported before. One possible reason is that previous studies have used 24B10 as a marker for all PR axons. 24B10 is expressed later than the δ-GAL4 and thus only in older PR axons, therefore the earlier overshoot event was not observed. We tried to test whether all R1-6 axons transiently overshoot but have not obtained conclusive result. Specific markers for R1/2/5/6 that are expressed early are needed. The R2/5/8-specific MT14-GAL4 unfortunately marks only more mature R2/5/8 (Supplementary Fig. 4).

**Figure 5.** Loss of Htl in WG disrupted PR retinotopic distribution in OS. (A–D) Glial membrane is visualized by repo > tdCD4GFP (green). (E–G) WG membrane is visualized by WG > mCD8GFP (green). (A–G) 24B10 (anti-Chaoptin; magenta) stains mature PR axons. Anti-HRP (cyan) stains all PR cells and axons. The combination of 24B10 and HRP can distinguish young (HRP only) and old (both HRP and 24B10) axons. (A,C) 24B10 staining stays about 8 rows behind the anterior edge of HRP signal. (B–B” and D–D”) Optical cross section of OS at the position of dashed lines in (A) and (C), respectively. (B, B’) In the control group, glial membranes occupy the entire OS cross section, with the PR axons in the central region. Younger axons (HRP only) are at the basal region, while older axons (both HRP and 24B10) are at more apical region. (D, D’) When Htl is knocked down in all glia, the glial membrane in the central region of OS is lost, indicating a loss of WG membrane. The differential apical-basal distribution of old-young PR axons is lost. (E) In WG > mCD8-GFP, WG membranes colocalize with axons in the central region of OS. (F–F’) Younger axons (HRP only) are at the basal region, while the older axons (both HRP and 24B10) are at more apical region and colocalized with WG membranes. (G) When Htl is knocked down in WG by the WG-GAL4, the WG membrane is reduced, and the differential apical-basal distribution of old-young PR axons is lost (H). (I,J) Statistics for the percentage of 24B10 axons in the basal region of OS. (I) repo > tdCD4GFP + HtlRNAi; compared with repo > lacZ (*p < 0.05). (J) WG > mCD8GFP + HtlRNAi compared with WG > lacZ (**p < 0.001). Two-tailed of Menwhitney analysis were used. Scale bar is 20 μm for (A–G).
phosphatase PTP69D, the Jak/STAT pathway and the kinase Pelle are all required for proper lamina termination of R1-6 axons. Whether these genes act on the retraction of the transiently overshoot axons or changed the targeting specificity from lamina to medulla is not clear.

Glia in the target field also plays a role in the R1-6 termination in lamina. In glia cell missing (gcm) and gcm2 double mutant, lamina gliogenesis is affected. In addition, ubiquitin protease Nonstop (Not) is required for the migration of the epithelial, marginal and medulla glia to their proper positions in lamina. Lamina glia migration can be affected by the JAB1/CSN5 subunit of the COP9 signalosome complex acting in PRs. Mutations or malfunction of these molecules all affected lamina glia and resulted in the R1-6 overshoot phenotypes, suggesting a role of lamina glia in R1-6 axon targeting. Whether the transient overshoot and retraction of R3/4 axons is also regulated by lamina glia is not clear.

WG is important for the retinotopic map formation. Lamina glia are critical for the proper R1-6 axon termination in lamina to form the lamina plexus. Whether the retinal basal glia affects retinotopy is not known. Here we show that WGs in eye discs non-autonomously affect the retinotopic projections of PR axons in OS and in lamina. When Htl is reduced, the apical-basal distribution of old-young PR axon projection in the OS is disturbed, and the proper termination of R1-6 axons in the lamina and the formation of lamina plexus is also disturbed. Since the retinal WG membrane does not extend to the lamina plexus, how do WG affect the R1-6 termination at lamina plexus presents an interesting question. Recent study has shown novel ability of WG to synchronize the differentiation of lamina neuron through the secretion of insulin-like peptides. It is possible that WG affects the R1-6 termination at lamina plexus by secreted molecules.

**Methods**

**Drosophila stocks.** Fly stocks are maintained at room temperature (RT). For all experiments, the flies are raised at 25 °C unless otherwise indicated. Special conditions were indicated individually. Fly stocks from Bloomington Stock Center are: repo-GAL4 (BDSC#7415); m60.5-GAL4(BDSC#41782 and #41795, on chromosome II and III, respectively); GMR74E02-GAL4 (BDSC #48320); hsFLP; UAS > STOP > myr-smGdP-HA UAS > STOP > myr-smGdP-V5 UAS > STOP > myr-smGdP-FLAG (BDSC #64085); Mz97-GAL4; hsFLP122; USA > STOP > mCD8GFP (BDSC#5366); yw; UAS-htl-DN; UAS-htl.DN (BDSC#6978); C527-GAL4; UAS-hid; tub-GAL80(III) (BDSC#7019).
**UAS-Bdl-RNAi (V4806)** is from VDRC and also gift from Dr. Yong Rao. **sev181-GAL4** (kindly provided by Chi-Hon Lee)\(^7\); **Htl-RNAi** (kindly provided by Christian Klämbt)\(^4\); **UAST-myr-GFP-V5-P2A-H2B-mCherry-HA** is an unpublished gift from Yung-Heng Chang and Joshua Dubnau (Department of Anesthesiology, Stony Brook University); **UAS-Kaede.K22** (kindly provided by Ann-Shyn Chiang)\(^3\); **UAS-hid, UAS-rpr** (gift from Suewei Lin); **UAS-CD2-HRP** (kindly provided by Tzu-Yang Lin); **Ro-tau-lacZ** (kindly provided by Philip A. Barker).

**Figure 7.** Killing of wrapping glia caused defect in retinotopic projection. The death genes **hid** and **rpr** were expressed by **WG-GAL4** with **tub-GAL80** (WG\(^{ts}> hid + rpr\)). (A–D) WG\(^{ts}> lacZ** served as control. (E–F) After 12 hrs temperature shift to 30 °C to induce death genes expression, the eye-brain complex is examined immediately. The basal WG layer showed significant increase of the apoptotic marker cleaved caspase 3 signals (E, green) and decrease of **Cut**\(^+\) WG cell number (F, red; quantitative analysis in I). Small **Cut**\(^+\) puncta, in contrast to the nuclear staining and probably representing debris of apoptotic WG cells can be detected in the middle region of the **WG > hid + rpr** eye disc (F). (C,G) In the optic stalk, the younger axons (24B10 alone, magenta) showed differential apical localization of in the control group (C), but in **WG > hid + rpr** are more disorganized in the optic stalk with more basal localization (G; quantitative analysis in J). (D,H) In the optic lamina, the retinotopic projections in lamina plexus are more disorganized in **WG > hid + rpr** (H) as compared with the control group (D). (K) Quantification data of the number of holes and the number of large holes (arbitrarily defined as holes with perimeter \(L_t > 20 \mu m\)). Scale bars are 20 \(\mu m\) for all.
Clonal induction. *Mz97-GAL4* flp-out clones were generated using hs-FLP122; *Mz97-GAL4; UAS > CD2 > UAS-mCD8GFP*. MCFO clones were induces by hs-FLP G5 and driven by GMR74E02-GAL4. In both experiments, larvae of mixed ages were heat-shocked for 10 minutes in 38 °C water bath. Larvae were raised for another 12 hr. (for *Mz97-GAL4* flp-out clones) or 48 hr. (for MCFO clones) after the heat-shock and larvae at late third larval stage were dissected to examine the clones. The glial flp-out clones were generated by heat shock of *repo > hs-Flp122;+/+; UAS-mCD8-GFP* for 15 min, and the eye disc was dissected 24 hr. after heat shock for ex vivo culture.

Conditional inactivation of GAL80<sup>ts</sup>. The flies after mating were raised at 17 °C (permissive temperature). When 12 hr before late 3rd larval stage, the larvae were shifted to a nonpermissive temperature (30 °C) to induce death genes expression for the indicated time.

Immunohistochemistry and confocal microscopy. The eye-brain complex was dissected from late third instar larvae and fixed in 4% EM-grade paraformaldehyde (PFA, Electron Microscopy Sciences, 30525-89-4) in 1X PBS (phosphate buffered saline) for 20-25 minutes and washed three times in 1xPBS. Primary antibodies were: mouse anti-2B10(CUT) (DSHB, 1:50); mouse anti-24B10 27 (DSHB, 1:200), anti-HA (4C12 mouse Ab, Abcam ab1424; 1:200), anti-FLAG (anti-dykddddk rabbit Ab, Sigma Aldrich, F2555; 1:200), anti-V5 (rat Ab, Novus Biologicals #NBP1-06712; 1:200) and rabbit anti-Cleaved Caspase-3 (Asp175, 1:200, Cell Signaling). Secondary antibodies used were Alexa-488, Cy3, Alexa-647 and HRP-Alexa-647(Jackson Immunoresearch, mouse and rat minimal cross-talk versions). All antibodies were diluted by PBST with 10% (v/v) normal goat serum (Jackson Immunoresearch). All images of fixed samples were acquired by Zeiss LSM 780 with the Plan-Apochromat 40x/1.4 oil objective. For live imaging and UV photoconversion of Kaede, LSM 710 inverted microscope was used with C-Apochromat 40x/1.2 W korr objectives. Optical sections were 0.2 (for 24B10/HRP staining), 0.8 and 1.2 μM thickness for fixed and live samples, respectively.

Image processing and analysis. 3D images were reconstructed and analyzed by the commercial software IMARIS 8.4.1 or 9.0.0 (Bitplane, Germany). XY figures are all presented as whole Z-stacks processed by IMARIS unless otherwise indicated. Cross and longitudinal sections were shown in partial Z-stack projections (2.5–10 μM). For live imaging, images were recorded every 10 minutes for 10–16 hrs. To analyze axonal movement, images were cropped into smaller region that only covers OS. IMARIS threshold cutoffs of 13.5 and 7.5 were used for green channel and red channel respectively to get rid of global background signals and make the outline of the OS clearer. The “Measurement points” function was used to define the length from top to bottom.
and from top to center of axons in OS around 40 time points. To analyze 24B10 distribution in optic stalk in Fig. 5, OS sections were cropped by IMARIS. Next, volumes of 24B10 and HRP staining were obtained after 3D surface rendering. The region covering the OS was then divided equivalently along Z axis. Percentage of 24B10 volume in apical region and basal regions were recorded. To analyze the size of hole in Fig. 7, the perimeter of the holes was measured.

**Ex vivo tissue culture and confocal microscopy.** Eye-brain complexes were cultured *ex vivo* as described previously.\(^{45,22}\)

**Transmission electron microscopy (TEM).** The larvae were cut into halves by dissecting scissor to reduce the sheared damage on the tissues. The head cuticle was gently removed by scissor to expose the eye-brain complex. The dissected eye-brain complex was quickly fixed in fixative containing 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate (Sigma), pH 7.4 in 4 °C for overnight. The tissues were washed three times (15 minutes each) with 0.1 M sodium cacodylate, 2% OsO\(_4\) (Electron Microscopy Sciences) in 0.1 M sodium cacodylate in RT for 1 h, three times in Milli-Q water and switched to 2% uranyl acid (Polysciences) for 30 mins in RT, then washed by Milli-Q water, and dehydrated by a series of different concentration of ethanol (50% once, 70% once, 80% once, 90% once, 95% once and 100% three times, propylene oxide twice; 15 minutes for each series dehydration). The dehydrated tissues then underwent a series of resin replacement in increasing ratio of Epon (EMBed-812 Kit)/propylene oxide (SIGMA-ALDRICH #471968) (1:3, 2:1, 1:1, 1:2, 1:5, then pure for each series dehydration). After dehydration, the tissues were baked in 60 °C oven for 48 hrs. 80 nm-thick sections were acquired with a diamond knife (Ultracut, Reichert-Jung, Vienna, Austria) and examined by TEM (Tecnai G2 Spirit TWIN, FEI Company, Hillsboro, OR) equipped with a Gatan CCD Camera (794.10.BP2 MultiScanTM). For visualization of HRP-expressing WG, Diaminobenzidine (DAB) staining are as described\(^ {73}\) but without Ni-intensified.

**Photoconversion of Kaede.** The photoconvertible fluorescent protein, Kaede\(^ {31-33}\), was used in this study. UV irradiation induces a peptide cleavage resulting in the rapid and irreversible conversion from green fluorescence to red fluorescence\(^ {35}\). The red signal slowly decays, while the green signal slowly recovers, probably due to newly synthesized Kaede protein. The green-to-red photoconversion was induced by 405 nm laser beam with 1.5–7% power of 25 mW laser (LASOS lasertechnik GmbH, LGN3001) for 300 hits (over 4.53 s; 0.01513 s/hit; without interval). The irradiating conditions were optimized (Supplementary Fig. 1). Low laser power and longer exposure work well in single PR irradiation. In order to reduce photoconversion in non-target cells, we only irradiated a single PR pair in a region smaller than 3 × 3 pixel\(^ 2\). The bleaching mode in Zen software was used to draw region of interests (ROI). Once the red-to-green ratio was raised, the activated region could be distinguished until the end of imaging period (9–16 hr). Due to the background photoconversion during dissecting and mounting, the red signals are clearer when overlaid with green signals.

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**Author Contributions**

Y.C.C. and Y.H.S. designed the experiment. Y.C.C. performed the experiments, processed and analyzed the data. C.K.T. prepared ex vivo imaging in Supplementary Figure 5. Y.C.C. and Y.H.S. wrote the manuscript. All authors reviewed the manuscript.

**Additional Information**

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**Competing Interests:** The authors declare no competing interests.

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