An axon scaffold induced by retinal axons directs glia to destinations in the Drosophila optic lobe

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Summary

In the developing Drosophila visual system, glia migrate into stereotyped positions within the photoreceptor axon target fields and provide positional information for photoreceptor axon guidance. Glial migration conversely depends on photoreceptor axons, as glia precursors stall in their progenitor zones when retinal innervation is eliminated. Our results support the view that this requirement for retinal innervation reflects a role of photoreceptor axons in the establishment of an axonal scaffold that guides glial cell migration. Optic lobe cortical axons extend from dorsal and ventral positions towards incoming photoreceptor axons and establish at least four separate pathways that direct glia to proper destinations in the optic lobe neuropiles. Photoreceptor axons induce the outgrowth of these scaffold axons. Most glia do not migrate when the scaffold axons are missing. Moreover, glia follow the aberrant pathways of scaffold axons that project aberrantly, as occurs in the mutant dachsous. The local absence of glia is accompanied by extensive apoptosis of optic lobe cortical neurons. These observations reveal a mechanism for coordinating photoreceptor axon arrival in the brain with the distribution of glia to multiple target destinations, where they are required for axon guidance and neuronal survival.

Key words: Glia, Drosophila, Axon, Migration

Introduction

Migration is a striking example of complex cellular behavior that is essential to development, wound healing and cellular immunity. In the nervous system, cell migration is a common step in the final positioning of neuronal and glial cell bodies prior to the assembly of neural circuitry. In the developing mammalian brain, neuronal precursors migrate along radial and tangential pathways to arrive at stereotyped destinations in the developing telencephalon (reviewed by Marin and Rubenstein, 2001). Schwann cell precursors migrate along axon tracts into the peripheral nervous system prior to ensheathing axon fibers (Carpenter and Hollyday, 1992). Given the central role of migration in neural development, it is not surprising that migration defects are common in human diseases of cortical organization (Ross and Walsh, 2001). Nonetheless, the mechanisms by which migrating cells choose specific pathways and locate appropriate destinations are not well understood.

Drosophila melanogaster has been a favorable model system in which to study cell migration. Genetic analysis has been applied to the mechanisms controlling movement of follicle cells around the developing oocyte (reviewed by Montell, 1999) and gonadal precursors to the site of the developing gonad (Deshpande et al., 2001; Stein et al., 2002). In the nervous system, there are many instances of neuronal and glial migration from sites of cell birth to the locales of developmental and mature function, both in the CNS (Klambt et al., 1991; Klambt, 1993) and PNS (Giangrande, 1994; Choi and Benzer, 1994; Sepp et al., 2000).

It is evident that the migration of neural cells to proper destinations can be a complex process, even in the relatively simple nervous system of the fruit fly. The adult Drosophila visual system contains glia of many distinct types and properties that are localized to specific sites in the retina and optic lobe (Tix et al., 1997; Saint Marie and Carlson, 1983; Trujillo-Cenoz, 1965). Some glia subtypes arise from progenitors located at sites well removed from their mature positions. Retinal basal glia arise from progenitors located in the optic stalk, the epithelial tube that connects the developing eye and brain. Basal glia precursors migrate to the basal surface of the retina (Choi and Benzer, 1994; Rangarajan et al., 1999). In the optic lobe, lamina epithelial and marginal glia migrate to their specific layers from progenitor zones located at the prospective dorsal and ventral margins of the ganglion (Perez and Steller, 1996; Huang and Kunes, 1998). This migration depends on local signaling from the photoreceptor axons, as it largely fails to occur in mutants that do not produce photoreceptor neurons (Perez and Steller, 1996) or are deficient in the activity of the COP9 signalosome (Suh et al., 2002). Conversely, lamina glia are required for the guidance of photoreceptor axons, and provide an essential stop signal for the R1-R6 subset of photoreceptor axons to terminate their outgrowth in the lamina (Poeck et al., 2001). These complex processes are controlled with precision in the three-dimensional milieu of the developing brain by mechanisms that remain unclear.

An important aspect of glial cell migration is the targeting of particular subtypes to specific layers of the developing ganglia. Given their common origin in dorsal and ventral margin progenitor zones, selective mechanisms would be

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needed to direct glia to different destinations. We show that specific highways for migration are provided by axons that originate in the vicinity of glial progenitors. Photoreceptor axons induce the outgrowth of these ‘scaffold axons’ upon their arrival in the brain, and targeted migration follows. These observations describe a cellular mechanism for the control of optic lobe glial migration by photoreceptor axons, an important element in the coordinated assembly of the precise neural architecture of the optic lobe.

Materials and methods

Fly stocks

All strains were grown on standard cornmeal medium (Ashburner, 1989). The following strains and transgenic animals were used in this study: wg-lacZ; so1; hh1; dpp-GAL4, UAS-CDS::GFP; omb-GAL4, UAS-CDS::GFP; ds1; ds2; ds-lacZ; ey1; UAS-Ras1N17.

Immunocytochemistry

Third larval instar-staged animals were examined immunocytochemically essentially as described by Kunes et al. (Kunes et al., 1993). Primary antibodies were used at the following dilutions: mouse anti-Repo 1:10, goat FITC anti-HRP (Cappel) 1:200, rabbit anti-β-gal (Promega) 1:500, mouse anti-glial cells missing 1:100, monoclonal rabbit anti-human caspase 3 (BD PharMingen) 1:100 and rat anti-Elav 1:25. Secondary antibodies were used at the following dilutions: Cy3 or Cy5-goat anti-mouse (Jackson Immunnochemo, Inc.) 1:100, Cy3 or Cy5-goat anti-rabbit (Jackson) 1:500, HRP-conjugated goat anti-mouse IgG (Jackson) 1:100. Specimens were viewed on a Zeiss LSM510 META confocal microscope.

Mosaic analysis and crosses

Mosaic analysis was carried out as described by Xu and Rubin (Xu and Rubin, 1993). Larvae were subjected to heat shock at 37°C for 5-7 minutes 24-36 hours after hatching to induce expression of an hsFLP transgene. After growth at 20°C, larvae were dissected at late third instar stage and processed for immunohistochemical analysis. The following crosses and strains were used in the experiments described:

(1) Identification of scaffold axons and temporal expression of Wg neurons: wg-lacZ/Y*CyO.

(2) Marker analysis of wg-domains: (A) y,hsFLP122; wg-lacZ/Y*CyO X y,w; UAS-CDS::GFP; tuba>γY,Cy2>GAL4/Y*CyO; (B) Omb-GAL4, UAS-CDS::GFP/FM7 X wg-lacZ/Y*CyO; (C) Omb-GAL4, UAS-CDS::GFP/FM7 X ds-lacZ/Y*CyO; (D) Dpp-GAL4, UAS-CDS::GFP/TM6B, Tb X wg-lacZ/Y*CyO; (E) Dpp-GAL4, UAS-CDS::GFP/TM6B, Tb X ds-lacZ/Y*CyO; (F) Repo-GAL4, UAS-CDS::GFP/TM6B, Tb X UAS-CD8::GFP; and (F) Repo-GAL4, UAS-CDS::GFP/TM6B, Tb X UAS-Ras1N17.

(3) Dependence of scaffold axon induction on retinal innervation: (A) so1; wg-lacZ/Y*CyO X so1/Y*CyO; and (B) eyD X wg-lacZ/Y*CyO. Retina development was assessed in both so1 and eyD animals by examining ommatidia formation in the eye disc. For each specimen, regional brain development was assessed with respect to normal, or a lack of, correlated eye development.

(4) Analysis of scaffold axon outgrowth: (A) ds1; wg-lacZ/Y*CyO X ds1/Y*CyO; (B) ds33k; wg-lacZ/Y*CyO X ds33k/Y*CyO; (C) y,hsFLP122; wg-lacZ/Y*CyO X y,w; UAS-Ras1N17; tuba>γY,Cy2>GAL4; UAS-CDS::GFP.

(5) Analysis of optic lobe apoptosis: (A) y,hsFLP122; wg-lacZ/Y*CyO X y,w; UAS-Ras1N17; tuba>γY,Cy2>GAL4; UAS-CDS::GFP; and (B) so1; wg-lacZ/Y*CyO X so1/Y*CyO; (C) wg-lacZ/Y*CyO.

(‘>’ indicates the position of an FRT site in the respective construct.)

Quantitative analysis of apoptotic cells

Quantification of apoptotic cells in sine oculis and wild-type animals relied on carefully matched confocal and developmental parameters. Optical slices (1.3 μm) were compared for age-matched specimens at a similar focal plane for all specimens analyzed. The number of apoptotic cell profiles in 50 μm² regions both proximal to the medulla neuropile and within the medulla cortex of sine oculis and wild-type animals were counted. Cortical neuron size (~3 μm) and overall brain size did not differ between sine oculis and wild-type animals, so cell counts were not biased by any intrinsic differences in cell sizes between these two genetic backgrounds. Differences in the number of neuropile-proximal and cortical apoptotic cells in sine oculis animals relative to wild-type animals were evaluated for significance using Student’s paired t-test.

Results

Axons from dorsal and ventral optic lobe organizing centers establish divergent pathways for glial migration

The Drosophila optic ganglia are derived from a pair of neuroectodermal proliferative centers known as the inner and outer anlage, which are located on the ventrolateral surface of each larval brain hemisphere (Green et al., 1993; Hofbauer and Campos-Ortega, 1990; Nassif et al., 2003) (reviewed by Meinertzhagen and Hanson, 1993). We have previously described the onset of Wingless (Wg) expression in a few cells located at the presumptive dorsal and ventral margins of the disk-shaped outer anlagen in young larvae (Fig. 2A) (Kaphingst and Kunes, 1994). These two ‘Wg domains’ are positioned as adjacent wedges within the disk-shaped outer anlage (Figs 1, 2). In mid-metamorphosis, the disk opens at a fissure located between the two Wg domains (yellow bar in Fig. 1A); the disk unfurls linearly, such that the Wg domains move to the future dorsal and ventral poles of the ganglion. Interestingly, the Wg domains are roughly coincident with sites revealed by clonal analyses to be the origins of glia that migrate into the lamina neuropile (Perez and Steller, 1996). We sought to investigate this relationship in greater detail.

We used a marker of wingless expression, the lacZ reporter construct 17en40 (or wg-lacZ) (Kassis et al., 1992) to examine Wg expression with respect to glial cell development and movement. In cells expressing wg-lacZ, cytoplasmic β-galactosidase fills cellular processes and permits the visualization of their detailed morphology. We observed that the paths of glial cell migration coincided with a stereotyped pattern of cytoplasmic extensions emanating from wg-lacZ expressing cells (Fig. 1C,D,F, arrowheads). The extensions followed stereotyped routes and terminated at specific destinations in the lamina, medulla and lobula. Glia formed chains along these extensions and accumulated in neuropile destinations beyond the extension’s termini (Fig. 1D). Marker analysis indicated that the glia undergo differentiation as they progress along these paths. Lamina glia express the early glial differentiation factor glial cells missing (gcm) (Hosoya et al., 1995; Akiyama et al., 1996; Egger et al., 2002) at their points of entry onto the extensions (Fig. 1E, white cells between arrowheads). Further along the path toward the neuropile, glia commence expression of the homeodomain protein Repo (Xiong et al., 1994) a marker downstream of Gcm expression. Thus, in the case of lamina marginal glia shown in Fig. 1E (Gcm expression) and Fig. 1D (Repo expression), differentiation can be assessed relative to their progression along the migratory pathway.
Figure 1. Axons from Wg-expressing neurons form pathways for glial migration to different optic lobe target layers.

(A) Schematic diagram of developing optic lobe architecture and glial migration viewed from a lateral perspective in a late third instar larval stage brain. Dorsal is upwards and posterior rightwards. Inner chiasm glia (Xi, red), medulla neuropile glia (MNG, purple), marginal and epithelial lamina glia (Ma/Ep, yellow), and lobula neuropile glia (LoG, light blue) exit the Wg domains (Wg, dark blue) via distinct scaffold axon fascicles. The eye disc (ed), photoreceptor axons (R-cell) and optic stalk (os) are indicated. The medulla (med, dark green), lamina (lam, light green) and lobula (lob, brown) neuropiles are indicated. (A') An enlarged schematic view of the ventral Wg domain, showing the distinct scaffold axon pathways for different glial cell types.

(B) Optic lobe architecture and glial migration viewed from the horizontal perspective. Distal is upwards. The scaffold axon fascicles are distributed on the proximal distal axis. The pathway for lamina glia is the most distally positioned, while the tract for lobula neuropile glia is most proximal. Labeling as in A. (B') An enlarged view of the ventral Wg domain in horizontal view, illustrating the lamination of scaffold axon fascicles and glial cell types. (C) Two scaffold axon fascicles (orange arrowheads) are observed extending from each of the dorsal and ventral Wg domains (Wg; dorsoventral boundary indicated by the orange line in C-F), which are visualized by expression of wg-lacZ (anti-β-gal; blue) in this late third instar stage brain. The two pairs of scaffold axon fascicles visible in this plane extend to the medulla neuropile (med n'pil, tract for MNG glia) and lobula neuropile (lob n'pil, tract for lobula neuropile glia). The neuropiles are visualized by anti-HRP antibody staining (green). The location of medulla cortex (med cortex) is indicated. (C') Image in C showing only wg-lacZ expression, to permit visualization of the scaffold axon fascicle pairs (orange arrows).

(D) Glia (anti-Repo staining; red) seen during migration (white arrowheads) from the Wg domains (wg-lacZ visualized by anti-β-gal staining; blue). The layer of lamina marginal glia (Ma) is visible in this plane, as well as some inner chiasm glia (Xi) headed towards a more proximal destination. (D') Image shown in D with only wg-lacZ expression shown in order to detail the scaffold axon fascicles (orange arrowheads).

(E) On their pathway towards the lamina (lam), glial differentiation is marked by the onset of Gcm expression (anti-Gcm, white, cells between arrowheads). The glia emerge from a region in which Optomotor Blind (Omb; anti-Omb, purple) is expressed under Wg control, and continue to express Omb as they migrate toward neuropile destinations. The area demarcated by white arrowheads, in which most cells express Gcm, can be compared with the onset of Repo expression in the same region, indicated by white arrowheads in D. (F) Scaffold axon fascicles (orange arrowheads) originate from a subset of Wg expressing neurons (wg-lacZ; anti-β-gal staining; white), which co-express the neuronal marker Elav (anti-Elav; red). Neuronal populations and neuropiles are labeled as in C. Scale bar: 20 μm.

The wg-lacZ positive extensions are evidently axons. Their cell membranes were labeled by anti-horseradish peroxidase antibody, a neuronal marker (Fig. 1C) (Jan and Jan, 1982). Their cell bodies were labeled by Elav, also a neuronal marker (Fig. 1F) (White et al., 1983). The axons extending toward the same neuropile destination were bundled together in a fascicle, as indicated by labeling of individual axons in mosaic animals (described below; see Fig. 4). Four wg-lacZ labeled fascicles were resolved emerging from each of the dorsal and ventral Wg domains (Fig. 1A,B). One fascicle from each domain...
extended to the border of the lamina field, terminating at a position adjacent to layers of glia known as the lamina epithelial (Ep) and marginal (Ma) glia [established nomenclature (http://www.flybrain.org, Accession Number PP00003)] (Tix et al., 1997). These layers of glia lie, respectively, above and below the layer the axon termini of the R1-R6 photoreceptors. Owing to the absence of specific markers, we could not distinguish epithelial and marginal glia prior to their separation into distinct layers. It seems likely, however, that both glial types migrate on the same pathway. One fascicle from each Wg domain extended to the cortex, neuropile boundary of the medulla, and was associated with the chain-like migration of medulla neuropile (MNG) glia. One fascicle from each domain extended to the boundary between the medulla and lobula, and corresponded to a pathway for migration of inner chiasm (Xi) glia, which demarcate the border between medulla and lobula neuropiles (Tix et al., 1997). The final pair of fascicles extended into the lobula neuropile (Fig. 1F) and formed a pathway associated with lobula neuropile glia (LoG). We will refer to these four sets of putative migratory guides as ‘scaffold axons’. Notably, the migration of these four glia types depends on retinal innervation of the optic lobe, a requirement that is explored below.

Earlier stage specimens were examined in order to determine the temporal relationship between glial migration and the outgrowth of scaffold axons. During the first two larval stages, most neuroectodermal cells of the outer anlagen express the cell adhesion protein Fasciclin 2 (Fas2), with the exception of those in the two Wingless domains; Wingless-expressing cells are Fas2 negative (Kaphingst and Kunes, 1994; Dumstrei et al.,

Fig. 2. Developmental profile of scaffold axon outgrowth, glial migration and retinal innervation. Wg expression commences in the mid-1st instar larval stage in a pair of domains in the outer anlagen, preceding retinal innervation by several days. Scaffold axon fascicle elaboration was visualized by wg-lacZ expression (blue in A-E or white in A’-E’). Glia were visualized by anti-Repo staining (red in all panels). Neuronal cell bodies and neuropiles were visualized by anti-HRP staining (green in A-E). Above each optic lobe image, which shows a different time point (youngest at left in A), is a schematic diagram indicating the state of ommatidial development in terms of the number of ommatidial columns. The developing optic lobe is outlined in white in A-E and yellow in A’-E’.

(A,A’) An early 2nd instar stage specimen, lacking any retinal innervation (0 columns), in which the two Wg domains contain a small number of cells. A small island of Repo-positive glia are observed in the prospective photoreceptor axon target region (arrowhead). Scaffold axon fascicles are not detected. (B,B’) A mid-2nd instar specimen, a time point just prior to ommatidial development. The anlage have grown in size, but scaffold axon tracts are not yet detectable. (C,C’) A late 2nd instar specimen in which one ommatidial column is detected in the retina, and a small amount of retinal innervation has occurred. The medulla neuropile has just begun formation. Scaffold axon fascicles are clearly visible emanating from the Wg domains and extending towards the neuropile (green arrowheads in C’). There is an increase in glia number within the neuropile region at this time point (yellow arrowhead). (D,D’) An early 3rd instar stage animal in which three columns of developing ommatidia were present in the retina. Scaffold axon fascicles begin to elaborate an architecture reminiscent of the late third instar (compare with Fig. 1C), in which tracts for medulla neuropile glia and lobula neuropile glia are distinguishable (green arrowheads in D’). Considerably more glia are visible in the neuropile region (yellow arrowhead in D’) in the focal plane shown. (E,E’) A mid-3rd instar stage specimen in which seven columns of ommatidia were detected in the retina. Scaffold axon fascicles for MNG glial migration are indicated (green arrowheads in E’).
2003). As development proceeds to the third instar stage, the neuroectodermal populations mostly convert to blast cells that produce the neurons and glia of the lamina and medulla. The differentiation of wg-lacZ positive scaffold neurons and the extension of their axons follow this general temporal scheme (Fig. 2). As seen in Fig. 2A,B, a small population of glia precedes the arrival of the first photoreceptor axons in the target field of the retinal axon. Perez and Steller (Perez and Steller, 1996) also described a small population of centrally located glia that preceded the arrival of photoreceptor axons in the lamina. At these early time points, when the Wg domains consist of fewer than a hundred cells; scaffold axon fascicles were not detected. Photoreceptor axons subsequently arrive in the temporal order that follows the posterior to anterior pattern of eye development. The elaboration of wg-lacZ positive scaffold axons was first detected as the first photoreceptor axons arrived in the target field, when only the first one or two columns of ommatidia had initiated differentiation in the eye disc (Fig. 2C). As retinal innervation continued, the number of glia in the neuropile regions increased steadily (Fig. 2D,E). Thus, Wingless-positive cells, though present long before the arrival of photoreceptor axons in the brain, appear to first extend axons when retinal axons begin to arrive in the brain.

The wg-lacZ labeled-axons also were examined in pupal stage animals, where mature axon projections into the optic lobe neuropiles could be resolved (Fig. 3). wg-lacZ positive axons could still be detected extending from cell bodies located at dorsal and ventral cortical positions. The axons projected to respectively dorsal and ventral targets in the medulla and lobula neuropiles (Fig. 3A,B). It is evident that the wg-lacZ-positive neurons include intrinsic neurons of the proximal medulla (Pm neurons), which extend arbors tangentially within small regions of the proximal medulla (Fischbach and Dittrich, 1989). Projections into a specific tangential layer of the lobula were also observed. Projections into the lamina neurpil were not observed; at the third instar stage the extensions terminate at the border of the developing lamina neurpil. These axons thus may not have become part of lamina circuitry, or alternatively have ceased wg-lacZ expression at the pupal stages examined.

**Clonal analysis defines the origins of glia and the neurons that guide their migration**

We sought to determine the location of progenitors that give rise to the distinct types of migratory glia and the neurons that formed their migratory pathways. The Wingless expressing cells of the dorsal and ventral domains are located in areas of complex gene expression controlled by Wingless (Wg) signaling activity (Fig. 4A) (Kaphingst and Kunes, 1994; Song et al., 2000). Adjacent to the Wg domains are non-overlapping cell populations that express the TGF-β family member Decapentaplegic (Dpp; Fig. 4C,E). Both the Wg- and Dpp-positive cell populations express the transcription factor Optomotor Blind (Omb; Fig. 4D) (Poeck et al., 1993; Huang and Kunes, 1998; Song et al., 2000). Dachsous (Ds), a Cadherin family member (Clark et al., 1995), is expressed in a graded fashion with respect to the Wg domains (Fig. 4D,E) (Song et al., 2000). These three genes, though expressed in different patterns, are under the control of Wg activity (Song et al., 2000).

By performing clonal analysis with tissue labeled to provide positional landmarks, it was possible to localize glial progenitors and scaffold neurons to distinct sites of origin (Fig. 4B, Table 1). The FLP/FRT system (Golic and Lindquist, 1989; Xu and Rubin, 1993) was used to generate somatic clones that were positively marked by membrane-bound GFP (UAS-CD8:GFP) (Lee and Luo, 1999). Rare recombination events were induced such that most specimens harbored only one or a few labeled cell clones in the developing optic ganglia, which were examined in late third instar larvae. As reported by Perez and Steller (Perez and Steller, 1996), clones including lamina marginal and epithelial glia were found to label progenitors

![Fig. 3. Fate of Wg-expressing neurons in the mature optic lobe.](image)
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Table 1. Glial and scaffold neuron clone distribution

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<th>Location</th>
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<th>Neuron</th>
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<td>2</td>
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</table>

The clonal relationship and location of neurons and glia that originate from the Wg domains was examined by using the FLP/FRT method to generate rare, small mitotic clones marked by UAS-CD8::GFP transgene expression. Glia were identified with anti-Repo antibody. For each specimen, clones were scored as labeling glia only (Glia), neurons only (neuron) or a mixed population of glia and neurons (glia and neurons) and the location of each clone was identified in accordance with the domain map shown in Fig. 4A. In total, 78 specimens were analyzed.

located at the dorsal and ventral margins of the outer anlagen (Fig. 4F). Clones that included lamina epithelial glia, lamina marginal glia, medulla neuroepithelial glia, inner chiasm glia or lobula neuropile glia were all found within the domain of cells that express Wg, Omb and Ds (Domain I; Fig. 4A; Table 1). A majority of these clones contained both labeled scaffold neurons and glia (16/26); clones with only glia or glia were less frequent. In the majority of specimens in which glia were labeled, the clone extended into multiple domains and included Domain I. With rare exception, these larger clones also contained neurons. Thus, at the time that somatic recombination was induced (the mid-second instar, 75 hours AEL (after egg laying)), most progenitor cells retained the potential to produce both neurons and glia. When the specimens were analyzed with respect to the glial types that were labeled, an interesting pattern emerged with regard to the position of labeled progenitor cells in the Domain I region. Labeled progenitors for each of glial cell type appeared in distinct domains on the proximal, distal axis (Fig. 4B). For example, the lamina epithelial and marginal glial progenitors were found in a more lateral position (Fig. 4F) than medulla neuropile glial progenitors (Fig. 4G). Inner chiasm glial progenitors were observed in an even more proximal location (Fig. 4H). Hence, the progenitor domains for distinct glial types appear to be organized into a proximal distal stack within Domain I, as illustrated in Fig. 4B.

Labeled scaffold neurons were most often included in the glial clones, and were found in close proximity to glial progenitors that used the axons as migratory guides (Fig. 4J). In a minority of cases, small clones were recovered which included only scaffold neurons (Fig. 4K). These clones were contained within Domain I (Wg, Omb, Ds expression) in all but two of nineteen cases. Our data do not resolve when the glial and neuronal lineages diverge. However, they do permit the conclusion that glia and the neurons that appear to establish their migratory pathways are generated in close proximity and with a lineage relationship.

Photoreceptor axons induce the outgrowth of scaffold axons
The migration of glia from the prospective dorsal and ventral margins of the developing optic lobe depends on the arrival of photoreceptor axons in the target field (Perez and Steller, 1996; Huang and Kunes, 1998). When photoreceptor axons are absent, as occurs in mutants that eliminate ommatidial development (sine oculis, eyes absent and eyeless) most glia remain stalled in their progenitor domains (Perez and Steller, 1996). When photoreceptor axons innervate only part of the lamina field, glia migrate to the region that receives retinal innervation. It has thus been supposed that photoreceptor axons attract glia into the lamina target field. We thus thought it might be informative to examine the glial migratory scaffold under conditions where glial migration did not occur.

To this end, the axon scaffold was examined in sine oculis

Table of contents

PDF

Development 131 (10)

Development 131 (10)
Fig. 4. Wg domain regions that give rise to glia and the neurons that guide their migration. (A) The area surrounding the Wg domains was divided into several subdomains on the basis of gene expression patterns. All of the genes categorized (omb, ds, dpp) are regulated by Wg activity. The schematic diagram shows that the Wg domain can be divided into two subdomains (I and III) on the basis of ds expression. Wg-expressing cells all express omb, and in one area also express ds. The four subdomains shown are: I, wg/omb/ds; II, wg/omb; III, dpp/omb/ds; and IV, omb. The overlapping expression patterns of these genes are shown in color for the top domain, and in outline form in the bottom domain. The location of the lamina (lam) and lobula (lob) are indicated. An arrow indicates the position of the optic fissure, where the two Wg domains separate during pupal metamorphosis. (B) A horizontal perspective of the optic fissure, where the two Wg domains separate. An arrow indicates the position of the bottom domain. The location of the lamina (lam) and lobula (lob) are indicated. A map of the overlapping expression patterns of these genes is shown in color for the top domain, and in outline form in the bottom domain. The origin of lamina Ma and Ep glia (#1, yellow), medulla neuropile glia (#2, purple), inner chiasm glia (#3, red) and lobula neuropile glia (#4, light blue). The position of these progenitor sites forms a stack on the proximal (#4) to distal (#1) axis. (C) Animal harboring wg-lacZ positive fascicles extending toward glial migratory destinations. A map of glial subtype origin is overlaid onto subdomain 1, showing the origin of lamina Ma and Ep glia (#1, yellow), medulla neuropile glia (#2, purple), inner chiasm glia (#3, red) and lobula neuropile glia (#4, light blue). The position of these progenitor sites forms a stack on the proximal (#4) to distal (#1) axis. (C) Animal harboring wg-lacZ (anti-B-gal staining; blue) and a transgenic marker for dpp expression (dpp-GAL4, UAS-CDS::GFP; GFP expression is green). In this lateral view of a late third instar stage optic lobe, the non-overlapping expression of wg and dpp is evident. dpp expression is also found in the inner proliferation center of the lobula (lob). (D) A late third instar optic lobe, like that shown in C, but harboring a ds-lacZ reporter (anti-B-gal staining in grayscale) and a transgenic marker for omb expression, omb-GAL4, UAS-CDS::GFP (GFP expression is red). Ds expression intersects Omb expression, distinguishing subdomains II and IV from I and III. (E) A late third instar optic lobe, like that shown in C, but harboring a ds-lacZ reporter (anti-B-gal staining in grayscale) and a transgenic marker for omb expression, omb-GAL4, UAS-CDS::GFP (GFP expression is red). Ds expression intersects Omb expression, distinguishing subdomains II and IV from I and III. (F-K) Cloonal analysis was performed in order to determine the origins of particular glial subtypes, and the neurons that form their migratory pathways. Random clones were generated using the FLP/FRT system (see Materials and methods) and labeled by membrane-bound GFP (UAS-CDS::GFP, green in F-K). (F) A zone originating within a distal layer of subdomain I (wg/omb/ds) labels lamina glia (Ep, epithelial glia). (G) A zone originating in subdomain I in a slightly more proximal focal plane than in F labels medulla neuropile glia (MNG). The medulla neuropile glia are also labeled by anti-Repo antibody (red). This clone is in the dorsal Wg domain (dorsal towards the top). (H) Clones originating in a more proximal layer of subdomain I than is shown in F or G label inner chiasm glia (Xi). The particular clone shown also labels the scaffold neurons and axons that project along the glial migratory tract (between arrowheads). In this specimen, expression of wg-lacZ is shown in blue. Inset: Xi glia shown double labeled by anti-Repo (red) and repo-GAL4, UAS-CDS::GFP (green) in order to visualize their characteristic size and morphology, which permit these glia to be easily distinguished from many other glial cell types. (I,J) Clones often labeled both scaffold neurons and glia (see Table 1). In these two specimens, single clones labeled both lamina Ma glia (arrows) and a few neurons whose axons project along their migratory pathway (arrowheads). (K) A small (three-or four-cell) clone (green) within the Wg domain (subdomain I, blue) labels neurons (arrowhead) that extend scaffold axons. Scale bars: in C, 20 μm for C-I; in J, 20 μm for J,K. A white or yellow bar indicates the boundary between dorsal and ventral Wg domains in all panels.

Thusly establishing a necessary pathway for glial migration. Conversely, glial migration, elicited by retinal innervation directly, might establish a necessary pathway for scaffold axon outgrowth. Two approaches were undertaken in an attempt to resolve this issue. In the first, scaffold axons were eliminated by neuronal expression of activated Ras1 (Ras1N17) in order to determine whether glial migration would occur in their absence. In the second, mutant animals with misdirected scaffold axons were examined in order to determine whether migratory glia follow the aberrant axon projections. Both approaches indicated that scaffold axons are necessary as glial migratory guides.

In the first approach, we used the ectopic expression of a dominant negative Ras protein (Ras1N17) (Lee et al., 1996), which was found to autonomously block the outgrowth of scaffold axons (Fig. 6D,E). The UAS-Ras1N17 transgene was expressed in small somatic clones that were labeled by the co-expression of UAS-CDS::GFP. Occasional somatic clones of
Ras1N17-expressing cells within the Wg domain region resulted in failure of scaffold axons to extend toward glial destinations. By contrast, clones that labeled lamina epithelial, marginal or medulla neuropile glia did not affect their respective migration (data not shown). When the scaffold axons were absent, glia stalled prior to migrating into the neuropile regions, as they did in so1 animals (arrowheads in Fig. 6D,E). That is, glia did not migrate when the scaffold axons were missing, even though retinal axons were present in the target field. These observations indicate that the scaffold axon fascicles are required as migratory guides for glial migration.

In the second approach, we examined mutants lacking the function of the Cadherin family member Dachsous (Ds) (Clark et al., 1995). As described above, Ds is expressed in a gradient that decreases with distance from the Wg domains (Song et al., 2000) (Fig. 4D,E). Ds is highly expressed by neurons in the Wg domains but not by migrating glia. Ds can mediate homophilic binding, and so one might suppose that the Ds gradient could provide positional information for axon outgrowth within the developing neuropiles.

Scaffold axons did indeed project aberrantly in the mutants ds1 and ds33k (Fig. 6B,C) and glia lined these aberrant pathways and accumulated in abnormal destinations. In the ds1 homozygous animal shown in Fig. 6B, scaffold axons which should project toward the medulla neuropile and form a path for medulla neuropile glia (as shown in Fig. 6A) were found extending toward the lobula cortex. Glia formed a chain along the aberrant axons (Fig. 6B’ and Fig. 6A) and accumulated abnormally in the lobula, while the number that reached the medulla was reduced. In the ds33k homozygous animal shown in Fig. 6C, the dorsal scaffold axons bifurcate and appear to direct glia away from paths toward neuropile destinations. Mutations in combgap, a negative regulator of optic lobe ds expression (Song et al., 2000) resulted in similar axon misrouting and glia distribution abnormalities (data not shown). The correlation between patterns of scaffold axon misprojection and glia migratory paths is a further indication that scaffold axons serve as migratory guides.

The migration of medulla neuropile glia is required for cortical cell survival

Prior work revealed extensive apoptosis in the optic lobes of ‘eyeless’ mutants of Drosophila (Fischbach and Technau, 1984; Fischbach 1983). In the mutant sine oculis (so), mosaic analysis has revealed that extensive cell death in the optic lobe is due to the lack of so function in the retina and not the brain. These and additional observations have led to the conclusion that the optic lobe phenotype of sine oculis is due to lack of retinal innervation. The ‘trophic’ function of photoreceptor axons could be direct, via provision of a survival factor, or indirect, e.g. by eliciting the migration of glia that provide a survival factor. We explore these distinct hypotheses below.

To address the issue, wild-type and so1 animals were examined for the onset of apoptosis by their expression of activated caspase, which can be monitored in Drosophila with an antibody against the activated human caspase 3 protein (Baker and Yu, 2001). In third instar stage wild-type animals, few cortical cells are labeled by the anti-Caspase labeling (Fig. 7A’). By contrast, so1 third instar larval optic lobes displayed
Fig. 6. Evidence that scaffold axons are required for glia migration and direct glia along specific pathways. (A,A') Wild-type scaffold axon fascicles (anti-β-gal; grayscale) in a wg-lacZ late third instar optic lobe project in a stereotyped fashion toward glial destinations. MNG and XI glia (anti-Repo; red) are seen following along fascicles towards their appropriate destinations. A higher magnification view of the yellow boxed area in A is shown in A'. (B,B') In ds33k homozygous animals, scaffold axon fascicles project aberrantly, and the distribution of glia is correspondingly aberrant. A higher magnification image of the yellow-boxed region of B is shown in B'; glia (yellow arrowheads) on an abnormal trajectory are closely associated with the misprojecting scaffold axon fascicle (anti-β-gal; grayscale). (C,C') Scaffold axon projections are likewise aberrant in ds33k homozygous animals. In this specimen, dorsal scaffold axon fascicles bifurcate onto aberrant trajectories, and glia are likewise misdirected. The higher magnification image of the boxed region of C shown in C' reveals mispositioned glia (anti-Repo; red) in association with the misrouted scaffold axon fascicles (anti-β-gal staining in grayscale). (D,D') Random somatic clones were generated expressing both a GFP marker and an activated Ras protein (Ras1N17) that inhibits axon extension. wg-lacZ (anti-β-gal staining; blue) was used to mark the migratory axon scaffold. In the late third instar optic lobe shown, a clone encompasses part of the dorsal Wg domain (white outline in D and yellow outline in D'). Glia (anti-Repo, red), which do not express the UAS-Ras1N17 transgene, stalled at the edge of this Wg domain (yellow arrowheads in D,D'), much as they do in ‘eyeless’ mutant strains (see Fig. 5). Scaffold axon fascicles cannot be detected emanating from the dorsal Wg domain, but are clearly visible extending from the ventral Wg domain (yellow arrows); ventral glia migrate normally. (E,E') A specimen like that shown in D,D' in which somatic clones (white outline in E, yellow outline in E') express UAS-Ras1N17 (labeled by co-expression of GFP, green in E). Clones of particular interest encompass both the dorsal and ventral Wg domains. Scaffold axons are absent from both and glia stall at both the dorsal and ventral margins of the Wg domains (yellow arrowheads in E'). Scale bars: in A, 20 μm (for A-D,D',E,E'); in A', 8 μm (for A',B',C').

an increased number of caspase 3-positive cells throughout the medulla cortex (Fig. 7B). Putative apoptotic cells were concentrated in regions where glia were particularly few or absent. Caspase 3-positive cells were also particularly prevalent in cortical areas immediately adjacent to the neuropile (arrowheads in Fig. 7B'). As noted above, areas particularly deficient in glia also displayed an irregular neuropile structure. These observations were subjected to a quantitative analysis (see Materials and methods). Areas of 50 μm² in 1.3 μm confocal optical sections of the medulla cortex were counted for the number of caspase 3-positive cells. In wild-type animals, an average of 0.6 (±1, n=10) activated caspase-positive cells were found per 50 μm² area. No regional differences in the density of apoptotic cells were observed in wild-type animals. so1 specimens displayed an average of 5.5 (±2.9; n=37) apoptotic cells in 50 μm² areas adjacent to medulla neuropile regions that lacked medulla neuropile glia. Elevated apoptosis, an average of 6.5 (±5.2, n=37) activated caspase-positive cells, was also observed in distal cortical cell populations whose axons would normally innervate glia-starved regions of neuropile. By contrast, cell populations adjacent to glia-rich regions of medulla in the same so1 animals showed only 2.4 (±2.5, n=37) apoptotic cells per 50 μm², while in the corresponding distal cortical cell populations that innervate these glia-rich regions, only 2.5 (±3, n=37) apoptotic cells per 50 μm² were found. Thus, although so1 animals displayed an overall increase in caspase-positive cells, the frequency was significantly greater in cell populations that innervated glia-starved regions of neuropile. The results of this analysis are statistically significant: for medulla neuropile proximal regions within so1 animals a paired t-test analysis yielded a P-value of 1.3e-06 (comparing glia-poor and glia-rich regions), while the same statistical analysis comparing cortical regions yielded a P value of 4.8e-07. Our observations suggest that both local and long-range trophic cues are provided by glia to cortical neurons.

To determine whether the increase in apoptotic cells was due to the absence of retinal axons or the absence of glia, we used Ras1N17 expression to block the migration of medulla neuropile glia along the scaffold axons, as described above. In the specimen shown in Fig. 7C, a small clone of cells expressing the UAS-Ras1N17 and UAS-CD8::GFP transgenes is localized to the Wg domain region. Glia accumulated at the border of this region instead of migrating into locations adjacent to the medulla neuropile. Notably, photoreceptor innervation was not markedly affected in such animals. The
Fig. 7. A normal glial distribution is required for cell survival in the developing medulla. Cell death was compared in wild-type and so1 animals in relation to the distribution of glia. (A-A') A wild-type late third instar optic lobe is stained to reveal a normal organization of the medulla neuropile (med. n’pil, anti-HRP staining, green) and distribution of MNG and Xi glia (anti-Repo, red). Very few apoptotic cells are evident by staining with anti-activated caspase 3 antibody (blue in A,A', shown in grayscale in A’). (B-B’) In so1 animals, an increased frequency of apoptosis was detected in the late third instar optic lobe (see the text for details). In the specimen shown, retinal axons have innervated the dorsal (dorsal is upwards) optic lobe, resulting in a relatively normally organized medulla neuropile. The ventral optic lobe lacks innervation and glia accumulate in the vicinity of the Wg domain (white arrow in B’). Few glia are found in the ventral neuropile region. An increased frequency of anti-activated caspase 3-positive cells (blue in B and B’, grayscale in B”’’) is seen throughout the medulla cortex. More apoptotic cells are found in the ventral region (regions between white, yellow and blue arrowheads; see text for quantification), where glia are rare. (C-C”) An animal in which glial migration was blocked by expression of UAS-Ras1N17 in somatic clones (marked by co-expression of UAS-CD8::GFP (green) (see the legend to Fig. 6, and Materials and methods for experimental details). A Ras1N17-expressing clone in the dorsal Wg domain region has blocked glial migration into the dorsal optic lobe in this late third instar stage specimen. Glia (red) accumulate at the position of the yellow arrowhead shown in the boxed area in C. A locally increased frequency of activated Caspase 3-positive cells is visible (blue in C; grayscale in C’, shown alone in grayscale between arrowheads in C”’). Scale bar: 20 μm.

cortical area adjacent to this glia-deficient region displayed an increased number of anti-caspase 3-positive cells (arrowheads in Fig. 7”). Thus, the absence of glia, rather than photoreceptor axons, appears the more likely cause of extensive apoptosis and neural cell loss observed in eyeless strains of Drosophila.

Discussion

Glia and neurons are known to use one another’s extended cellular processes to guide their migration over long distances and through complex terrain. Well-known examples include the migration of cortical precursors along radial cell processes (Rakic, 1988) and the movement of Schwann cell precursors along axon tracts into the PNS (Carpenter and Hollyday, 1992). The directed migration of glia along axons has also been described in Drosophila (Choi and Benzer, 1994; Giangrande, 1994; Sepp et al., 2000). This mode of cell dispersal has thus been conserved across significant evolutionary distances.

We show in this report that Drosophila optic lobe glia use axon fascicles as migratory guides and that the extension of these axon fascicles is induced by the ingrowth of photoreceptor axons from the developing retina (Fig. 5). The migratory scaffold axons emerge from optic lobe regions that are in close proximity to sites where glial cells originate; both arise in the dorsal and ventral domains where cells express the morphogen Wingless (Figs 1, 4) (Kaphingst and Kunes, 1994). When the scaffold axons were eliminated by the autonomous expression of an activated Ras transgene, glia failed to migrate and stalled at the borders of their progenitor sites (Fig. 6). Extensive cortical cell apoptosis ensued (Fig. 7). When the scaffold axons projected aberrantly (in animals mutant for the cadherin Dachsous; Fig. 6), glia followed the aberrant routes to incorrect destinations. The longstanding observation that glial migration does not occur in eyeless mutant Drosophila (Perez and Steller, 1996; Huang and Kunes, 1998) might thus be explained by an indirect mechanism in which innervation controls the establishment of an axon scaffold necessary to direct glial migration.

The migratory scaffold axons were identified by their cytoplasmic expression of β-galactosidase from lacZ under the control of a wingless promoter (Kassis et al., 1992). The neurons are thus residents of the Wg domains, a point additionally supported by labeling small numbers of neurons that projected their axons toward glial destinations (Fig. 4). In total, four different wg-lacZ delineated pathways were identified. These appear to account for all the pathways taken by optic lobe glia that have been identified as migratory by clonal studies. Separate pathways were identified for medulla neuropile glia, lobula neuropile and inner chiasm glia. A single scaffold axon pathway was observed leading to the marginal and epithelial glial layers of the lamina, suggesting that both of these glial types follow the same pathway. Perhaps these glia become separated only on the interposition of photoreceptor R1-R6 growth cones as they arrive in the lamina. Whether the epithelial and marginal glia arise from distinct precursors that migrate on the same pathway is unclear. In all cases, glia were observed to form migratory ‘chains’ along axonal extensions,
resembling a similar organization of migratory glia on retinal axons en route from the optic stalk to the eye field (Choi and Benzer, 1994) and from midline progenitor sites to destinations in the PNS (Sepp et al., 2000). In pupal stage animals, the \( wg-lacZ \) labeled neurons were observed in dorsal and ventral cortical locations, sending projections into neuropile targets consistent with the patterns of glial migration. However, no axons from \( wg-lacZ \) positive neurons were observed extending into the lamina neuropile at this stage.

The optic lobe regions surrounding the Wg domains display complex patterns of gene expression, mainly because of the signaling activity of Wingless (Kaphingst and Kunes, 1994; Song et al., 2000). Our clonal analysis indicates that all five migratory glial cell types we examined arise from these domains (Fig. 4 and Table 1). Interestingly, the sites from which particular glia arise are stacked on the proximal distal axis (Fig. 4B) in a manner that correlates with target destinations in the developing ganglia. Thus, for example, somatic clones that label the medulla neuropile glia are located at a position that corresponds to the medial/distal position of MNG glia relative to the lamina and lobula glia. Furthermore, on the basis of their expression of \( wg-lacZ \), as well as clonal analysis (Fig. 4), the neurons that extend scaffold axons arise in close proximity to the sites of glial origin. Indeed, as somatic clones induced in mid-second instar larval animals often labeled both scaffold neurons and migratory glia, the glia and neurons must share common progenitors. It is curious that all of these distinct cell types express wingless. We have no evidence that axonally transported Wg functions in optic lobe development, as it does in the development of the neuromuscular junction (Packard et al., 2002). Partial elimination of \( wg^* \) activity (by the use of a conditional \( wg^* \) allele) did not result in a specific defect in glial migration in the optic lobe (R.D. and S.K., unpublished) but other possible functions of Wg were not addressed by this analysis.

Early studies on \( sine oculis \) mutants (Fischbach, 1983; Fischbach and Technau, 1984) demonstrated that lack of retinal innervation results in excessive optic lobe cell death, especially in the medulla, which is reduced to less than half of its normal volume without innervation (Power, 1943). Our analysis suggests that these earlier observations may be accounted for by a lack of glial migration. Though apoptosis was generally elevated in the medulla of animals lacking retinal innervation, a higher level of cell death was found in regions particularly devoid of medulla neuropile glia (Fig. 7). This effect could be produced with retinal axons present when glial migration was blocked by elimination of the scaffold axons (via Ras1\(^{N17}\) expression). Therefore, retinal innervation and its inductive effect on neuronal development in the lamina is not sufficient for survival. Glial migration is required. A role for glia in neuronal survival has also been documented in the lamina, where neuronal cell death follows the elimination of glia in animals carrying a visual system specific allele of \( repo \) (Xiong and Montell, 1995).

Our observations suggest a developmental mechanism for the control of glial cell migration that depends on the establishment of an axon scaffold for guidance of migrating glia (Fig. 8). In normal development, a small number of glia migrate into the target field of the photoreceptor axon prior to the arrival of the first photoreceptor axons (Fig. 2) (Perez and Steller, 1996). These glia, which migrate independently of retinal innervation, may serve a necessary early role in photoreceptor axon guidance. They may be targets for the first retinal axons to arrive in the optic lobe, and provide the first signals that differentiate the outgrowth termination points of the R1-R6 and R7/8 axons (Poeck et al., 2001). We propose that the first photoreceptor axons to arrive in the optic lobe elicit outgrowth of the scaffold axons from neurons at the dorsal and ventral margins. Subsequent migration of glia from the dorsal and ventral margin progenitors is then both permitted and directed along the specific pathways of the scaffold. After the erection of the migratory scaffold, glial migration may be independent of continued photoreceptor axon ingrowth (see the text). Through this mechanism, the distribution of glia throughout the optic lobe is coordinated with innervation by the photoreceptor axons.

![Fig. 8. A cellular model for the control of glial migration by retinal innervation. A small number of glia (red) migrate into the target region for photoreceptor axons prior to ommatidial development, and are ready to provide initial guidance cues to the first photoreceptor axons (green; top two panels). These early photoreceptor axons trigger the elaboration of the axonal scaffold for glial migration (blue; third panel from top), which extends in a stereotypical fashion to establish the multiple pathways (not depicted). Glia generated in the Wg subdomain 1 (yellow) can then migrate to target destinations, such as the lamina (shown, bottom panel). Subsequent migration may be independent of continued retinal axon ingrowth (see the text). Through this mechanism, the distribution of glia throughout the optic lobe is coordinated with innervation by the photoreceptor axons.](image-url)
at the dorsal and ventral margins of the optic lobe (Huang and Kunes, 1998). It seems that the induction of scaffold axon outgrowth by the photoreceptor axons may be direct, as the outgrowth occurs in the hedgehog mutant, hh¹, in which the first steps of lamina neuronal development fail to occur (Huang and Kunes, 1996). Thus, one might suppose that retinal axons emit a chemotactant for scaffold axon outgrowth, either synthesized in the retina or acquired from environmental sources and redistributed by retinal axons (e.g. as for Netrin) (Hiramoto et al., 2000).

The system for glial migration guidance we describe permits diversified cell types to originate from a common site, and yet target specific locations in complex neuropiles. One could imagine that as more complex and diversified neuropiles evolved, relatively simple changes in the developmental pathway of glial progenitors and the projections of scaffold axons would deliver glial support to new structures. This system also has the feature of functioning as a developmental ‘checkpoint’ that fine-tunes the general hormonal coordination of imaginal development. Thus, upon their initial arrival in the brain, retinal axons provide a fine level of local cellular control over the movement of glia, preparing the target field for the next steps of optic lobe development.

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2302 Development 131 (10)
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