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Generation and early differentiation of glial cells in the first optic ganglion of *Drosophila melanogaster*

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Summary

We have examined the generation and development of glial cells in the first optic ganglion, the lamina, of *Drosophila melanogaster*. Previous work has shown that the growth of retinal axons into the developing optic lobes induces the terminal cell divisions that generate the lamina monopolar neurons. We investigated whether photoreceptor ingrowth also influences the development of lamina glial cells, using P element enhancer trap lines, genetic mosaics and birthdating analysis. Enhancer trap lines that mark the differentiating lamina glial cells were found to require retinal innervation for expression. In mutants with only a few photoreceptors, only the few glial cells near ingrowing axons expressed the marker. Genetic mosaic analysis indicates that the lamina neurons and glial cells are readily separable, suggesting that these are derived from distinct lineages. Additionally, BrdU pulse-chase experiments showed that the cell divisions that produce lamina glia, unlike those producing lamina neurons, are not spatially or temporally correlated with the retinal axon ingrowth. Finally, in mutants lacking photoreceptors, cell divisions in the glial lineage appeared normal. We conclude that the lamina glial cells derive from a lineage that is distinct from that of the L-neurons, that glia are generated independently of photoreceptor input, and that completion of the terminal glial differentiation program depends, directly or indirectly, on an inductive signal from photoreceptor axons.

Key words: *Drosophila*, glial cells, lineage analysis, glial proliferation, visual system, optic lobe, cell-cell signalling.

Introduction

The developing optic lobes of the imaginal *Drosophila* visual system are influenced by interaction with the developing eye imaginal disc. In particular, the first optic ganglion, the lamina, depends on the retina for cues to regulate neurogenesis and proper structuring of lamina cartridges (Meyerowitz and Kankel, 1978; Fischbach and Technau, 1984; Selleck and Steller, 1991). The dependence of the developing lamina on proper eye development is illustrated in mutants with reduced or absent eyes (Power, 1943; Fischbach and Technau, 1984). In these cases, the underlying lamina is correspondingly reduced or absent, missing both neuronal and glial elements. The retina and optic lobes arise from distinct precursor cells set aside during embryogenesis, which come into contact during imaginal development (for reviews see Poulson, 1950; Meinertzhagen, 1973; Kankel et al., 1980). Cellular proliferation in these tissues begins during early larval life. In mid-third instar larvae, organization of the eye disc begins as a morphogenetic furrow moves across the disc in a posterior-to-anterior direction; cells posterior to the furrow differentiate into photoreceptor neurons and accessory cells (reviewed in Tomlinson, 1988; Ready, 1989; Rubin, 1989; Banerjee and Zipursky, 1990; Hafen and Basler, 1991). The photoreceptor (R) cells send projections across an epithelial sheath, the optic stalk, into the brain. A subset of the retinal axons (R1-6) terminates in the presumptive lamina (Trujillo-Cenoz, 1965). Retinal innervation is required to induce the terminal cell divisions that produce L-neurons (Selleck and Steller, 1991). It has been suggested that divisions of the lamina glial precursors do not coincide with the births of the L-neurons (Hofbauer and Campos-Ortega, 1990).

The studies mentioned above have emphasized neuronal components. In contrast, relatively little is known about the development of glial cells in the lamina. In this study, we investigated the influence of R-neurons on the early differentiation of the glial cells in the lamina. Two enhancer trap lines that express β-galactosidase in the lamina glial cells during larval life were used. These markers are not expressed in the absence of retinal innervation. The striking parallel between photoreceptor-dependent neurogenesis and photoreceptor-dependent glial differentiation led us to inquire whether a pluripotent progenitor cell exists that gives rise to L-glia as well as L-neurons. Genetic mosaics were used to investigate the lineage relationship between glia and neurons in the lamina. This analysis demonstrates that the glial cells arise from precursors that are distinct from the L-neuron precursors. Furthermore, pulse-chase

experiments using the thymidine analog BrdU indicate that many of the L-glia are generated several hours prior to, and therefore independently of, retinal innervation. L-glia continue DNA synthesis within the developing lamina following R-axon ingrowth. This synthesis occurs normally in genetic backgrounds in which R-axons fail to innervate the optic lobes. These results indicate that although retinal innervation is necessary for the normal development of both L-glia and L-neurons, the generation of glial cells proceeds independently of photoreceptor input.

Materials and methods

Stocks and strains

Flies were grown on standard cornmeal medium (Cline, 1978) at 18ºC or 25ºC. Canton S served as the wild-type strain. Mutants and balancer chromosomes were as described by Lindsley and Grell (1968). The glial marker, 3-109 (inserted on chromosome 3 at 94BC), was kindly provided by C. Klämbt and C. S. Goodman. A P element enhancer trap screen (O'Kane and Gehring, 1987) was also carried out in our lab: PZ, a plasmid-based transposon construct (P[ry^+, kan^R, $lacZ^+$], Mlodzik and Hiromi, 1992) was mobilized from the dominantly marked *Cy*O chromosome in PZ, *Cy*O/+; Δ*2-3*, *Sb*, *ry/ry* males; these were crossed to *ry/ry* virgins. Phenotypically wild-type offspring (ry^+, Cy^+, Sb^+), representing new insertions in germline cells, were used to found strains that were then screened for interesting or useful inserts. For this study, two inserts on the second chromosome, B380 and VP19, were used.

Immunohistochemistry of whole-mount brains

Brains were dissected from late third instar larvae in phosphate buffer (0.1 M sodium phosphate pH 7.2) and fixed in 2% paraformaldehyde for 30-60 minutes at room temperature or overnight at 4ºC. Samples were blocked in BSN [Balanced Salt Solution (BSS, Ashburner, 1989)/0.3% Triton X-100/10% goat serum] for 1-4 hours, and incubated at 4ºC overnight with primary antibodies. Samples were then washed with several changes of PBT (0.3% Triton X-100 in PBS), blocked as before, and incubated with secondary antibodies.

For detecting β-galactosidase enzymatic activity together with retinal axons, tissues were instead fixed for 2 minutes in 0.2% benzoquinone, washed thoroughly, and incubated at 37ºC overnight in staining solution (Simon et al., 1985) prior to the regular antibody procedure. Photoreceptors were detected with mAb24B10 (Zipursky et al., 1984).

Rabbit anti-β-galactosidase antibody (Cappel) was used at a dilution of 1:200; goat anti-HRP antibody (FITC conjugate, Cappel) was used at 1:500 to stain neuronal membranes (Jan and Jan, 1982). Rat anti-ELAV antibody (gift of K. White) was used at a dilution of 1:80 to detect a neuron-specific antigen, the ELAV protein (Robinow et al., 1991). mAb24B10 (gift of S. Benzer) was used at a 1:3 dilution. Other antibodies [mouse anti-BrdU (Becton Dickinson), goat anti-mouse Ig and goat anti-rabbit Ig (FITC or rhodamine conjugates from Cappel, horseradish peroxidase (HRP) conjugate from BioRad)] were used at a 1:100 dilution. HRP-conjugated secondary antibodies were developed with 0.5 mg/ml diaminobenzidine (DAB) and 0.004% hydrogen peroxide, yielding a brown precipitate. Specimens were mounted in 70% glycerol and examined on a Zeiss Axiophot microscope or viewed by confocal scanning laser microscopy (MRC 600, Biorad) and analyzed using the manufacturer's software.

Cryostat sectioning and immunohistochemistry of adult heads

Heads were severed under PBS, embedded in OCT (Tissue Tec), mounted for horizontal sectioning, and frozen on dry ice. Thick sections (12 μm) of heads were cut on a Reichert-Jung Frigocut 2800 cryostat. Tissues were then fixed in 2% paraformaldehyde, blocked for 30 minutes in BSN, and incubated with the appropriate antibody for 30 minutes. Sections were rinsed, reblocked, incubated with an HRP-conjugated secondary antibody, and developed with DAB and hydrogen peroxide as described above. In some cases, a second set of antibodies was then applied. To distinguish between the two antigens, the first round of enzymatic DAB development was performed in the presence of 0.03% each cobalt chloride and nickel sulfate, to give a black precipitate (Ashburner, 1989). Rabbit anti-β-galactosidase (Cappel) was used at a 1:2000 dilution and all other antibodies were diluted 1:100.

BrdU in vivo labelling

A stock of 100 mg/ml BrdU in 1:1 DMSO:acetone was diluted in acetone for topical applications. Final concentration for "short pulses" was 1 mg/ml; for "long pulses" was 50 mg/ml. The availability of applied BrdU can be estimated from the number of rows of cells in the eye disc that incorporate label. We found the BrdU was available for less than 0.5 hour with the low dose and more than 6 hours with the high dose. Two- to four-hour embryo collections were taken, synchronized as first instar larvae, and aged to third instar. Animals were taken at various timepoints, washed in PBT, blotted dry, and then treated batchwise with approximately 1 μl BrdU solution per animal. In some cases, it was necessary to verify that animals were "pre-innervation" with respect to retinal input. This was done by dissecting similarly aged animals and staining with anti-HRP antibody, to assay for the presence of photoreceptor axons. Treated larvae were transferred to fresh food and allowed to age 2-24 hours to late third instar stage. Whole-mount brains were incubated with anti-β-galactosidase primary antibody as described. Tissues were washed, then post-fixed for 10 minutes in 2% paraformaldehyde to protect the β-galactosidase antigen from subsequent acid treatment (2 N HCl in PBT for 30 minutes), which is required to expose the BrdU antigen. After washing and blocking as described, brains were incubated with anti-BrdU antibody, followed by appropriate secondaries. Secondary antibodies were preabsorbed against fixed and blocked wild-type brains to reduce background fluorescence.

BrdU in vitro labelling

This followed a modified protocol from Truman and Bate (1988). Third instar larvae were dissected in phosphate buffer and incubated in a 30 μg/ml solution of BrdU in Grace's medium (Gibco) for 30 minutes at 25ºC. Tissues were fixed and stained as above.

Genetic mosaics of the lamina

A strain carrying a widely expressed P element-*lacZ* reporter insert, VP19/*Cy*O, was crossed to a strain homozygous for the construct, P[ry^+, Δ*2-3*]99B, which encodes a stable source of P transposase (Laski et al., 1986; Robertson et al., 1988). Half of the progeny carry both transgenes: VP19/+; Δ*2-3*/+. The transposase is able to excise the P element insert during somatic mitosis at a variety of developmental times, creating a mosaic patch consisting of the cell in which the excision occurred and all of its progeny (but see Discussion). Such a patch stands out as $lacZ^-$ in a field of $lacZ^+$ cells when the tissue is analyzed for the presence of the β-galactosidase enzyme. Progeny were raised at 18ºC and collected as adults within 24 hours of eclosion. The heads of all mosaic candidates were cryostat sectioned and stained with anti-β-galactosidase antibody, using bis-benzimide (1 μg/ml) as a nuclear counterstain (Ashburner, 1989). Mosaic patches from the

central part of the lamina were scored on the basis of several serial sections to increase diagnostic confidence.

Results

Enhancer trap lines marking lamina glial cells

P element-based enhancer trap lines provide useful cell type-specific markers (O'Kane and Gehring, 1987). Two lines that show glial expression were employed for this study. The enhancer trap line 3-109, kindly provided by C. Klämbt and C. S. Goodman, expresses β-galactosidase in glial cells in the embryo and a variety of other stages (C. Klämbt and C. S. Goodman, personal communication). A second line, B380, was isolated in our laboratory. Fig. 1A-C show horizontal sections through 3-109 adult optic lobes, stained with anti-β-galactosidase antibody (brown nuclei). Stained cells within the lamina were identified as glia based on their characteristic position and by their failure to express a general neuronal antigen, the ELAV protein (Robinow and White, 1991). The position of *lacZ*-positive cells corresponds to the glial cells described in previous studies in several dipterans (Trujillo-Cenoz, 1965; Saint Marie and Carlson, 1983a,b; Shaw and Meinertzhagen, 1986). Two layers of cells expressing the 3-109 reporter lie above the lamina neuropil. They correspond to the satellite glia in the cortex, and the epithelial glia in the neuropil. A third layer of cells lies beneath the lamina neuropil at the medial border of the lamina, and corresponds to the marginal glia cells. In Fig. 1B (and magnified in Fig. 1C), sections are also stained with anti-ELAV antibody (black nuclei); the two antigens do not overlap. Together, these markers account for essentially all of the nuclei that are detected by bis-benzimide staining and that have been described in the adult lamina (Strausfeld, 1976). The reporter is also expressed in an additional layer between the retina and the lamina, named the subretinal cells. Finally, expression is detected in non-neuronal cells, presumably medullary glial cells, which surround the medulla neuropil.

3-109 marks lamina glial cells in third instar larvae

Given the specificity of the adult expression pattern, we asked whether 3-109 also specifically marks glia in the developing third instar lamina, at the time when cellular differentiation begins. We found that 3-109 is expressed in a restricted set of cells in the larval brain (Fig. 1D-F). Expression in the lamina region is continuous through pupal stages, allowing β-galactosidase-positive cells to be traced from the larval to the adult stage. This permitted accurate identification of glial cells well before their acquisition of a fully differentiated morphology. Fig. 1D shows a confocal micrograph of the developing lamina of a 3-109 climbing third instar larva. Retinal axons were visualized with anti-HRP antibody (Jan and Jan, 1982). Reporter expression is restricted to the innervated portion of the lamina. Expression is seen even in the most anterior region, implying that the onset of expression is coincident with R-axon arrival at the anterior margin (Meinertzhagen, 1973).

The termini of photoreceptors R1-6 are bounded by layers of glial cells just medial and lateral. These were identified as the marginal and epithelial glial layers by analogy to their description in *Musca* (Trujillo-Cenoz and Melamed, 1973; for *Drosophila* see Kankel et al., 1980). At this stage, note that the epithelial glial cell layer lies closer to the marginal glial layer than it does in the adult animal; as the lamina neuropil expands the nuclei of these two cell types become displaced from each other. The third layer of lamina glial cells is more lateral, and corresponds to the satellite glia. The lamina glia (L-glia) can be distinguished from the L-neurons based on the expression of either the 3-109 reporter or the ELAV protein. At this time, as in adults, these expression patterns do not overlap. In Fig. 1E and F, two focal planes of the same 3-109 third instar larval brain are shown labelled with anti-β-galactosidase and anti-ELAV antibodies. (Due to the curvature of the tissue, a single focal plane is insufficient to view all the pertinent cell types.) Note that the satellite glial nuclei are positioned between the lateral (L1-4) and medial (L5) lamina neurons. These panels also indicate β-galactosidase expression in the medulla glia, positioned between the marginal glial cells of the lamina and the underlying medulla neuropil. These medullary cells are distinguished from the L-glia not only by their position but also by the characteristic disc shape of their nuclei. Finally, expression is detected in the subretinal cells. At this time, most of the subretinal layer still resides in the eye disc, from which these cells are derived (Cagan and Ready, 1989); only a few cells have crossed to the lateral margin of the brain.

Expression of glial cell markers depends on retinal innervation

The generation of lamina neurons is known to depend on the ingrowth of retinal axons (Selleck and Steller, 1991). To determine whether events in lamina glial development are also dependent on retinal innervation, expression of the 3-109 reporter was examined in a *sine oculis (so)* mutant strain. This mutation results in eyes with a variably reduced number of photoreceptors, ranging from none to nearly the full complement. In adult *so* flies, the lamina is reduced in size, corresponding to the reduced size of the retina. The lamina phenotypes are strictly a consequence of defective eye development (Fischbach and Technau, 1984).

The expression pattern of 3-109 in a wild-type animal is shown in Fig. 2A. In *so;* 3-109 larvae which lacked photoreceptors, the reporter was not expressed in the developing lamina region (not shown). In larvae with partial retinal innervation, staining is limited to the immediate vicinity of the axons (Fig. 2B). This demonstrates that expression of the marker depends, directly or indirectly, on photoreceptor axon ingrowth.

We isolated an additional enhancer trap line, B380, which is also expressed in lamina glia, as well as in other cell types. As in the 3-109 line, reporter expression in the developing lamina is confined to the innervated portion. In *glass* mutants, photoreceptors project aberrantly to the developing brain, frequently innervating a reduced area of the lamina (Selleck and Steller, 1991). The expression of B380 was correspondingly reduced in these mutants, and was limited to the vicinity of the axons (not shown). Together with the previous experiment, this suggests that

the arrival of photoreceptor axons induces changes in glial cell gene expression.

The lamina glial cell lineage is distinct from the L-neuron lineage

Given the similarity between neuronal and glial marker expression upon retinal innervation, we asked whether lamina glia and neurons derive from common precursors. A mosaic analysis was undertaken using somatic excision of a P-element-based reporter gene. We used a ubiquitously expressed enhancer trap line, VP19 (Benson and Steller, unpublished). Mosaic patches were generated by inducing somatic excision of the P element in VP19 heterozygotes. Excision was driven by the Δ*2-3* construct, which encodes a somatically active P transposase (Laski et al., 1986; Robertson et al., 1988). Excision events occur in mitotically active cells. We expected excisions to be generated randomly with respect to time and space. We predicted that the majority of events would lead to the loss of VP19 reporter activity from a given cell; that cell's progeny would be almost always β-galactosidase negative (see Discussion). The half-life of the β-galactosidase protein is short (at most a few hours) relative to the period between the last cells divisions in the lamina and emergence of the adult fly (several days).

Serial horizontal thick sections of heads of VP19; Δ*2-3* heterozygous adults were examined for β-galactosidase expression patterns. In adults, the different cell types in the lamina can be identified by the position of their nuclei (see Fig. 1). Only in the extreme dorsal and ventral regions are some assignments difficult due to curvature of the tissue. Therefore, we relied on sections from the central part of the lamina for our analysis. Sections were also stained with bis-benzimide to confirm the positions of *lacZ*$^-$ cell nuclei.

In our study, somatic excisions occurred with high frequency: in the sixty-five optic lobes examined, forty-three showed excision events. Most patches of non-expressing cells were relatively small, containing fewer than 100 cells. In all cases, mosaic borders within the lamina clearly separated the neurons from the glial cells. Among these, several mosaic patches included neurons of both the lamina and the medulla, but excluded lamina glial cells. Two kinds of mosaics were particularly instructive: Fig. 3B shows a section from one individual in which all the L-glia have retained reporter expression, but L-neurons are unstained. Fig. 3C shows the opposite case, in which no expression is detected in glia, but nearly all neurons express the reporter. Taken together, these observations suggest the early separation of neuronal versus glial lineages in the lamina. Patches containing both L-neurons and L-glia were found only when the patch contained most or all of the cells in the optic lobe. Based on previous analyses of optic lobe proliferation, we believe that these large patches indicate a very early excision event (White and Kankel, 1978; Hofbauer and Campos-Ortega, 1990). A summary of our results is provided in Table 1A. These data indicate that neurons and glia in the lamina are readily separated by genetic mosaic analysis and therefore must derive from distinct groups of precursor cells.

Some of the mosaic patches included only one type of lamina glial cell. This suggested that the different glial types within the lamina might also derive from distinct precursors. To examine this more closely, a second mosaic analysis was carried out using the 3-109 marker as the target for somatic excision (Table 1B, and data not shown). Mosaic borders often separated the L-glia from glial cells outside of the lamina. When mosaic patches were observed within the lamina, their borders separated glial cell types, as distinguished by position of their nuclei. These observations suggest that L-glia are more closely related to each other than to other glia in the visual system, and that glial cells of a particular type are more closely related to each other than to other glial subtypes.

Lamina glia are generated prior to photoreceptor ingrowth

Given that L-neurons and L-glia derive from distinct lin-

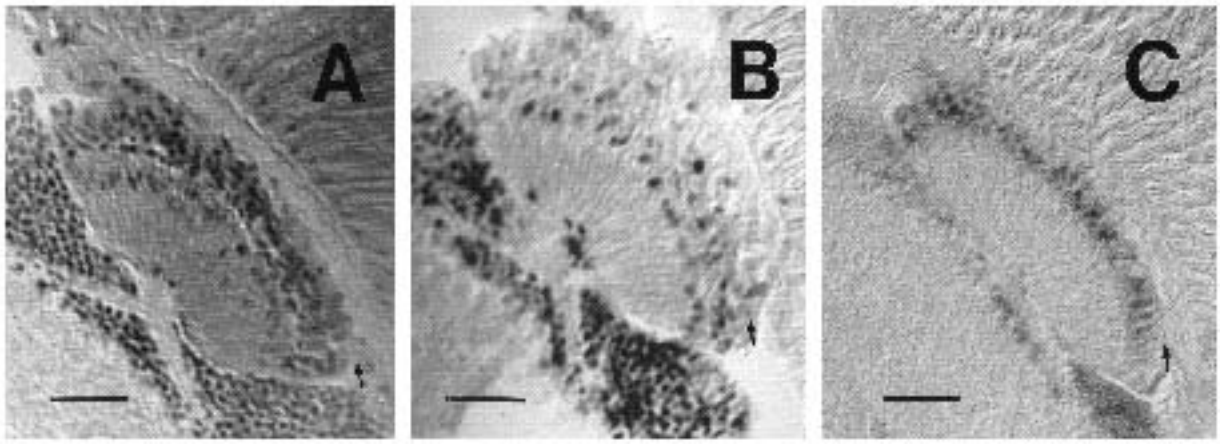

Fig. 3. Lamina mosaic analysis. Adult horizontal head sections were stained with anti-β-galactosidase antibody (dark nuclei). (A) Ubiquitous reporter expression in the parent strain, VP19. (B, C) Mosaic animals which carry the VP19 reporter and the Δ*2-3* transposase. An animal that has lost reporter expression in most L-neurons while retaining expression in L-glia is represented in panel B. An animal that has lost reporter expression in L-glia while retaining expression in most L-neurons is represented in panel C. Arrows indicate the border between the lamina (left) and the retina (right). Anterior is to the top of the page. Scale bars, 20 μm.

Table 1. (A) *Somatic mosaic analysis of lamina glia and neurons, using a ubiquitously expressed VP19 reporter as the target for excision*

Optic lobes examined	65
Total number of patches affecting optic lobes	56
optic lobes containing one lacZ-negative patch	30
optic lobes containing two lacZ-negative patches	13
Patches confined to the lamina	37
confined to lamina neurons	8
confined to lamina glia	29
crossing L-neuron to L-glia boundaries	0
Patches containing lamina and non-lamina cells	14
L-neurons plus medullary cells	10
Whole lamina plus medullary cells	2
Whole optic lobe	2
Patches not affecting lamina cells	5

(B) *Somatic mosaic analysis of glia, using the glial-specific reporter 3-109 as the target for excision*

Retina-lamina complexes examined	42
Patches scored	22
Patches segregating lamina from retina	14
Patches within the lamina	8

In each of these cases the patch was restricted to a single glial cell layer, representing a single glial cell type.

eages, it seemed possible that these two cell types would be generated at different times in development. Proliferative events that produce ganglion cells in the optic lobes have been described for Lepidopterans and Dipterans (Nordlander and Edwards, 1969a,b; White and Kankel, 1978; Hofbauer and Campos-Ortega, 1990). In order to determine more specifically when L-glia are generated, we performed a birthdating analysis in which S-phase cells were marked by incorporation of bromo-deoxyuridine (BrdU), a thymidine analog detectable by a monoclonal antibody (Gratzner, 1982; Truman and Bate, 1988). We devised a simple, rapid and efficient means of administering BrdU pulses in vivo. BrdU in a DMSO:acetone suspension was topically applied to third instar larvae. Viability is higher with this treatment than with injection, and adjusting the dose allows both short and long in vivo pulses and pulse-chases.

A series of pulse-chase experiments was conducted in which staged early to mid third instar 3-109 larvae (3IL) were pulse-labelled with BrdU and allowed to develop to the late third instar stage, when the glial reporter is first expressed in the lamina. The third larval instar lasts approximately two days. During much of the first day (early 3IL), extensive cell divisions resulted in substantial BrdU labelling throughout the optic lobes, as seen previously (White and Kankel, 1978; Hofbauer and Campos-Ortega, 1990). Fig. 4A-C and 4D-F show two examples of brains in which the larvae were pulsed approximately 15 hours after the beginning of 3IL stage, and chased to late 3IL. Brains were double labelled with anti-β-galactosidase antibody (green) to detect glia, and anti-BrdU antibody (red). Labelling is seen in both glial and non-glial cells. Within the glial cells, label from a short (< 30 minutes) pulse followed by a long chase appears in most cells of a given glial layer, where a layer corresponds to a glial cell subtype. This suggests synchrony within the precursors of a particular subtype. For a particular time, there is preferential incorporation into particular layers, distinguishing between precursors of different subtypes. Pulse-chases of similarly aged partially innervated *so*;3-109 larvae also showed label incorporated into L-glia (not shown, but see below). The pattern of incorporation was similar in innervated and non-innervated portions of the developing lamina. This suggests that the glial precursors divide normally in this mutant strain.

Toward the end of the first day of 3IL stage, synchronous BrdU incorporation into glial precursors ceases. Mid 3IL animals pulsed with BrdU just prior to R-axon entry and aged to late 3IL showed no label in the glial cells (not shown). Short pulses initiated after R-axons reach the brain (mid 3IL, post-innervation through late 3IL) occasionally labelled a few scattered cells, which were identified as glia by the expression of the 3-109 reporter (Fig. 4G), and efficiently labelled the lamina precursor cells (LPCs) just anterior to the developing lamina (Selleck and Steller, 1991). Pulse-chases initiated after R-axon entry traced the movement of BrdU from the LPCs into the body of the lamina, roughly in the shape of a column (Fig. 4H). The column of BrdU does not overlap with the glial marker, indicating that glial cells are not derived from LPC divisions. A summary of results of pulse-chase experiments is found in Table 2. From these experiments, we conclude that the generation of most glial cells occurs well before photoreceptor ingrowth, and that generation of lamina glial cells cannot be innervation-dependent. This further implies that the absence of reporter expression in non-innervated brains is not due to missing glial precursors, but instead reflects the lack of an inductive differentiation signal from the eye disc.

Post-innervation labelling in the glial layers

As was seen in Fig. 4G, short pulses of BrdU administered after R-axon arrival in the brain occasionally label lamina glial cells. Similar observations have been reported in the butterfly *Danaus* (Nordlander and Edwards, 1969b). Since it is not known whether glial cells in the lamina remain diploid, BrdU incorporation is not necessarily an indication of mitotic activity, but may reflect endoreduplications leading to polyploidy. At least some postembryonic insect glia are known to be polyploid (Nordlander and Edwards, 1969a; see also Robinow and White, 1991). In either case, scattered incorporation could be due to DNA replication in only some cells, or it could result from the loss of synchrony in a large population of different cells. To distinguish between these possibilities, we applied large doses of BrdU to late third instar larvae, thus providing continuous labelling throughout the chase period. If the majority of glial cells were still replicating DNA, but were no longer synchronized, then we expected a long pulse to label a large number of cells. In contrast, if scattered incorporation represented DNA replication in only some cells, then the number of labelled cells should not increase greatly.

Fig. 5A-C shows a 3-109 larval brain that was labelled continuously for 6 hours during late 3IL stage. The column of non-glial BrdU-positive cells within the lamina marked the products of LPC divisions (compare with Fig. 4H). The layers of glial cells were also labelled, indicating extensive

Table 2. *Summary of pulse chase data. Short BrdU pulses were administered at various times, followed by chases to late third instar*

Time of pulse	Number of animals tested: wild-type	Number of animals tested: mutant	Cells proliferating at time of pulse
late second or very early third instar (3IL)	12	7	central brain and optic anlage: labelling of L-neuron and L-glial precursors
early 3IL	12	15	central brain: few cell divisions occurring in the optic anlage
early to mid 3IL	12	14	glial precursors undergo synchronous divisions as layers
mid 3IL, pre-innervation	11	1	optic anlage divisions continue; glial precursors are not labelled
mid 3IL, post-innervation, to late 3IL	11	6	asynchronous incorporation begins in glial layers. L-neuron precursors undergo final divisions

DNA replication after the onset of 3-109 reporter expression. This demonstrates that asynchronous DNA replication continues in most, if not all, L-glia.

It remained possible that continued DNA replication in glial cells takes place in response to some signal from the photoreceptors or from the developing L-neurons. If DNA replication in the glial cells required retinal innervation, then continuous labelling of non-innervated late 3IL brains would show no BrdU incorporation into the lamina. A *so*;3-109 brain that was labelled continuously for 6 hours is depicted in Fig. 5D, with the lamina region indicated. This lamina had received no retinal input. Therefore, expression of the 3-109 marker was not detected, and the LPC division products were absent (compare with Figs 4H and 5C). There were, however, cells showing BrdU incorporation in the presumptive lamina. We believe these correspond to glial cells because they are arranged in layers rather than columns. The incorporation of BrdU into such layers was also seen in larvae of another eyeless mutant fly, *eyes absent* (Sved, 1986). Regardless of whether this incorporation is associated with glial cell divisions, or represents glial polyploidization, this process clearly does not depend on retinal innervation.

Discussion

Innervation-dependent differentiation of glial cells

Power (1943) was among the first investigators to demonstrate a correlation between the number of ommatidia in the adult retina and the volume of the first optic ganglion. Subsequent studies have emphasized the dependence of optic lobe development upon proper innervation from the eye disc (e.g. Meinertzhagen, 1973; Meyerowitz and Kankel, 1978; Fischbach and Technau, 1984; Selleck and Steller, 1991). These previous studies have primarily focused on the development of the neuronal components of the lamina, yet, the iterative modular structure of the lamina also permits detailed analysis of other components at the level of single cell types.

In the present work, we have examined the influence of retinal innervation on the glial cells of the lamina. We have used two enhancer trap lines, 3-109 and B380, which mark lamina glia (L-glia). Expression of β-galactosidase in the lamina of these lines begins immediately after the arrival of R-axons in the brain, and continues to adulthood. The induction of these markers progresses along the posterior-anterior axis concomitantly with the arrival of additional axons. No expression is seen in eyeless individuals, and expression is proportionally reduced in brains that receive reduced retinal input. The mutations used in these experiments, *sine oculis (so)* and *glass*, are known to autonomously affect eye development (Fischbach and Technau, 1984; Meyerowitz and Kankel, 1978; Moses and Rubin, 1991). We conclude that the induction of these markers in L-glia depends, directly or indirectly, on retinal input.

A similar situation has been described for neuronal markers in the lamina. In this case, the terminal divisons generating L-neurons depend on retinal innervation, accounting for their absence in eyeless mutants (Selleck and Steller, 1991). In contrast, glial precursor cell proliferation proceeds apparently normally (see below). Therefore, although some aspects of L-glia differentiation appear to require signals from the eye, the birth of these cells is independent of eye development.

We have not determined whether expression of glial reporters in the lamina depends on interaction with photoreceptors directly, or whether it is mediated by other cells. However, two observations are consistent with the former hypothesis. One is that the photoreceptors come into close contact with glial cells, which are present in the presumptive lamina (Trujillo-Cenoz and Melamed, 1973; see also Fig. 1D). Additionally, the 3-109 reporter, unlike neuronal antigens, is detected in the medial glial layers immediately upon the arrival of R-axons (data not shown). Therefore the response of these glia to the arrival of R-axons in the brain is very rapid. In a similar system, ingrowth of antennal neurons to the antennal lobe of the brain of a moth induces glial invasion and morphologic changes (Tolbert and Oland, 1989). Initial events in this induction require only sensory axon input and response of neuropil-associated glia. Participation of antennal lobe neurons is not essential (Oland et al., 1990).

The fate of glial precursors in the absence of innervation

The quantitative correspondence between the size of the eye and the lamina in adult specimens of various visual system defective mutants (Power, 1943) stems from at least two causes. First, lamina neurogenesis does not proceed in the absence of photoreceptor ingrowth (Selleck and Steller, 1991). Second, non-innervated regions of the developing lamina degenerate, beginning in very early pupal stage (Fischbach and Technau, 1984). We assume that lamina glial precursors are included among the degenerating cells, for two reasons. First, adult eyeless flies have no remnant of lamina cartridges, nor extra cells in the region (Power, 1943; Fischbach, 1983), indicating loss rather than trans-

formation of precursor cells. Second, degeneration extends from the lateral margin to the medulla neuropil, suggesting that all cell types in this region are dying (Winberg and Steller, unpublished).

The use of somatic excisions to study lineage relationships

P-element-based *lacZ* reporters are convenient cell-autonomous markers, which can be employed for mosaic analysis by inducing somatic chromosome loss, recombination or P element excision events (Laski et al., 1986; Robertson et al., 1988). Creating genetic mosaics via somatic excision of the P transposon offers several advantages. First, the marker can be located anywhere in the genome. Second, the frequency of mosaics is high enough that large quantities of data can be collected. In our analysis, 66% of optic lobes examined were mosaic. Third, excision events can apparently take place at a variety of developmental times and, with our markers, do not seem to affect viability of the animal. This allows examination of small clones in adult tissue. The major drawback that we have encountered, ironically, is the high frequency of excisions: many animals had two or three small non-contiguous patches, which we believe represent multiple events. This reduces the resolution with which the data can be analyzed in these animals. There is also a low probability that the P element transposon will be reinserted rather than lost. In the majority of these cases, we anticipated that the new insert would not be expressed in the lamina. In our experience, fewer than 1 in 20 germline transpositions generated show lamina expression (Benson, Berthon, Chadwick, Perez, Ressler, Shannon, Wiesbrock, Winberg and Steller, unpublished observations). Even less frequently does an insert show preferential expression in a particular lamina cell type. Therefore, we believe that these potentially misleading events would be quite rare and not affect our major conclusions.

Distinct origin of L-glia and L-neurons

Previous studies indicate that ganglion cells of the lamina and outer medulla are derived from the same primordium, called the outer optic anlage (Nordlander and Edwards, 1969b; White and Kankel, 1978). It is not known at what point various cells in this primordium take on restricted fates. In our analysis of genetic mosaics, forty-seven out of fifty-one patches affecting the lamina (92%) contained either glia or neurons but not both. The patches that contained both cell types were quite large, encompassing the entire lamina as well as other regions of the optic lobe. This indicates that L-glia and L-neurons are not very closely related. In ten cases, patches included medulla neurons with lamina neurons. If these patches represented single events, this would indicate that neurons of these two ganglion layers are more closely related to each other than to their glial neighbors.

The precursors of particular glial cells have been identified in only a few cases. In *Drosophila*, the longitudinal glia of the embryonic ventral ganglion are derived from glioblasts, large cells that divide symmetrically to increase in population and which eventually give rise exclusively to glial cells (Jacobs et al., 1989). In the developing CNS of grasshopper embryos, Doe and Goodman (1985) reported the existence of glial precursors, distinct from neuroblasts, although both are formed in the midventral neuroepithelium. In contrast, the subretinal glial cells and other non-neuronal support cells of the developing eye disc derive from pluripotent precursors (Ready et al., 1976; Cagan and Ready, 1989). In some vertebrate systems, commitment of precursors to neuronal or non-neuronal fates takes place early (Bronner-Fraser and Fraser, 1989; Raff, 1989; Hall and Landis, 1991). However, other workers have identified progenitors capable of giving rise to both cell types (Wetts et al., 1989; Turner et al., 1990; Frank and Sanes, 1991). We propose that lamina glia are generated by a distinct set of glioblasts, which is set aside from neuronal precursors, and which is ultimately derived from the outer optic anlage.

Proliferation of glial precursors

Precursor proliferation and generation of particular cells in the optic lobes of insects has been investigated by Nordlander and Edwards (1969a,b). Others have examined histogenesis in the optic lobes of *Drosophila* (e.g. White and Kankel, 1978; Hofbauer and Campos-Ortega, 1990; Selleck and Steller, 1991), but in these studies, few labelled cells were clearly identified as glia.

We have found three general periods during which BrdU pulses are incorporated into lamina glial lineages. The first corresponds temporally to the proliferation of optic lobe neuroblasts in late first instar and second instar larvae. Nordlander and Edwards (1969b), and White and Kankel (1978), observed symmetric divisions of neuroblasts during these stages. It is possible that similar divisions of glioblasts are taking place as well.

The second period of efficient BrdU incorporation into glial precursors is in the first half of third instar larval stage, before retinal axons grow into the optic lobe. At this time, a large number of optic lobe precursor cells divide in the outer proliferation center (OPC, White and Kankel, 1978). This proliferation center contains neuroblasts and ganglion mother cells that give rise to neurons in the lamina and outer medulla; we suspect that it contains glial precursors as well. We have observed that BrdU applied at this time is preferentially incorporated, such that a particular layer of glial cells is almost completely positive or almost completely negative (see Fig. 4A-F). Recalling that layers correspond to different glial subtypes, this suggests that various glial subtypes are generated at slightly different times. Later, a subset of OPC neuroblasts at the anterior margin of the developing lamina gives rise to lamina precursor cells (LPCs); their terminal divisions, which produce L-neurons, are induced by photoreceptor ingrowth (Selleck and Steller, 1991). Significantly, BrdU incorporated in this domain does not chase into L-glia. We conclude that unlike L-neurons, lamina glial cells are not derived from LPCs.

Finally, glial cells within the region of the lamina already innervated by photoreceptor axons continue to show asynchronous BrdU incorporation. This DNA synthesis takes place in mutant animals completely lacking photoreceptors and L-neurons, and therefore apparently does not depend on signals from the eye. It is possible that this incorporation represents the onset of polyploidy, which is common in Dipteran cells. All uptake of label into the lamina stops

by the end of the first day of pupal stage (Hofbauer and Campos-Ortega, 1990).

Other instances in which neurons and glia of a single tissue are born at different times have been reported. In the hawkmoth *Manduca sexta*, antennal-lobe glia proliferate after the formation of the antennal neuropil, generating cells that enclose glomerular units (Oland and Tolbert, 1989). Most chick dorsal root ganglia neurons are born before most glia (Carr and Simpson, 1978). Similarly, birthdates of neurons in the rat superior cervical ganglion generally precede those of glia (Hall and Landis, 1991).

Differences between glial subtypes in the developing lamina

Structural examination of the glial cells of adult Dipterans has shown that morphological subtypes are arranged in fixed layers (Trujillo-Cenoz, 1965; Strausfeld, 1976; Saint Marie and Carlson 1983a,b; Stark and Carlson, 1986). We have found that these subtypes are distinct from very early in their development. First, using somatic excisions to detect mosaicism within the lamina glial population, we frequently observed patches that included cells of only one glial subtype. This suggests an early separation within the glial precursor population, such that precursors may become restricted to generate L-glia of a certain layer or subtype. Second, during the proliferation of glial precursors, BrdU is incorporated in a layer-by-layer fashion. This indicates that cells within a glial lineage undergo their S-phases synchronously.

Interaction of glia and neurons during development of the lamina

In the mature lamina, several roles have been suggested for the glial cells. Specialized structures known as capitate projections may enable epithelial glia to provide nutrients to L-neurons (Trujillo-Cenoz, 1965; Saint Marie and Carlson, 1983a,b; Stark and Carlson, 1986). Support for this possibility comes from the recent demonstration that R-neurons degenerate if they are unable to make connections with the optic lobe (Campos et al., 1992). Adult lamina glial cells are physically inserted between cartridges and between compartments such that they could serve as insulators (Saint Marie and Carlson, 1983a,b; see also Tolbert and Oland, 1989, 1990; Steindler et al., 1990). Finally, a possible developmental role for L-glia could be to provide R-axons with information about their target field. At the time that individual photoreceptor axon fascicles reach the presumptive lamina, their particular target cells have not yet been born (Selleck and Steller, 1991). Yet, axons of photoreceptors R1-6 terminate in the lamina, while R7-8 continue into the medulla (see Figs 1D, 2A). It is possible that the glia mediate this decision, through transient interactions with photoreceptors (Trujillo-Cenoz and Melamed, 1973). Interestingly, although the glial cells are not fully differentiated at this time, they may be partly functional. Partially differentiated cells have been reported to influence optic nerve projections in vertebrates. Chick optic axons rely on contact with a neuroepithelial substratum for correct projection to their target area (Silver and Rutishauser, 1984); in rats, this neuroepithelium eventually gives rise to optic nerve glia (reviewed in Raff, 1989).

Conclusions

We have examined early events in the generation and differentiation of glial cells in the first optic ganglion of *Drosophila*. This work demonstrates that the differentiation of glial cells in the lamina depends, directly or indirectly, on photoreceptor axon ingrowth. In contrast to L-neurons, lamina glia are generated independently of photoreceptor input and are derived from a distinct lineage.

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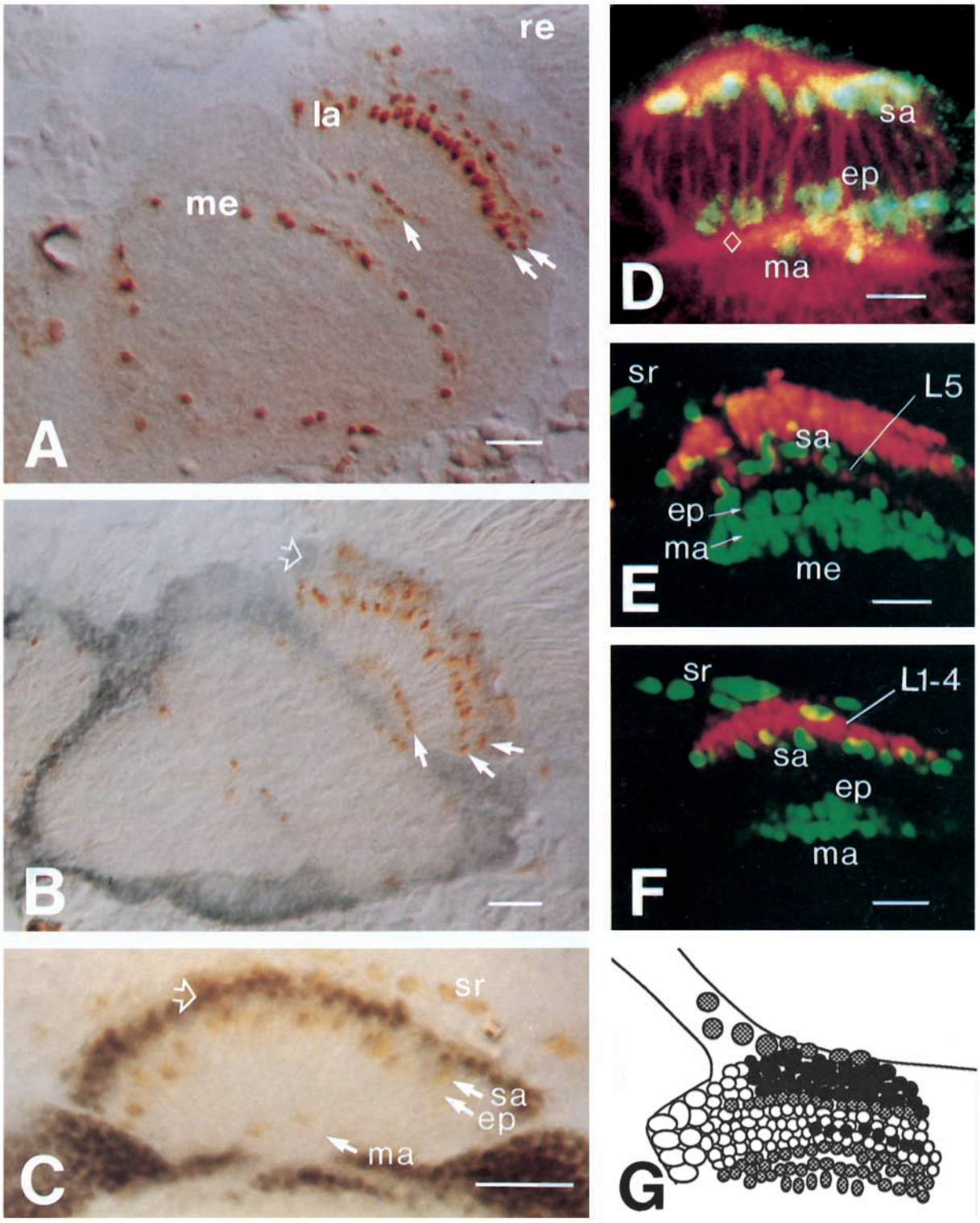

Fig. 1. Expression of 3-109 in wild-type animals. (A) Adult head section showing relative positions of the retina, lamina and medulla. Glial nuclei (brown) are detected with anti-β-galactosidase antibody, L-glia are indicated with arrows. (B, C) Adult head sections stained with anti-β-galactosidase antibody (brown nuclei) to detect glia and anti-ELAV antibody (black nuclei) to detect neurons. L-neurons are indicated with an open arrow, L-glia with closed arrows. (D-F) Confocal micrographs of whole-mount, third instar larval brains stained with anti-β-galactosidase antibody (green nuclei) to detect glia, and with either anti-HRP antibody (in red, panel D) to visualize photoreceptor axons or with anti-ELAV antibody (red nuclei, panels E, F) to detect neurons. The layer of photoreceptor 1-6 termini is indicated by a diamond in panel D. (G) A schematic representation of a third instar lamina, with glial nuclei in gray and neuronal nuclei in black. Anterior is up in A and B. Panel D shows a frontal section with lateral up. Anterior is to the left and lateral is to the top of the page in E-G. ep, epithelial glia; la, lamina; L1-4, L5, lamina neurons; ma, marginal glia; me, medulla glia; re, retina; sa, satellite glia; sr, sub-retinal cells. Scale bars, 20 μm.

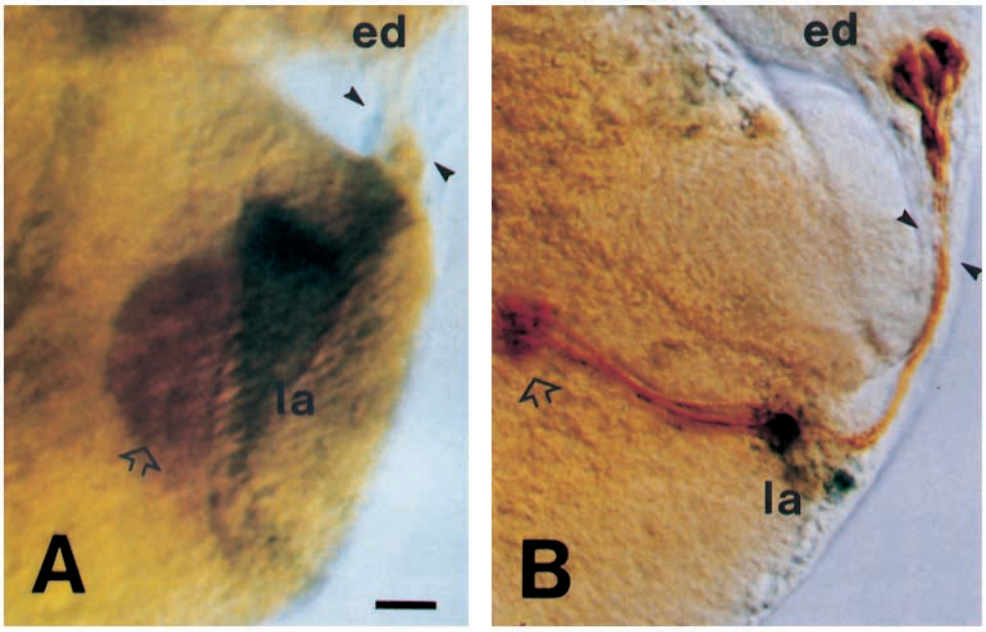

Fig. 2. Innervation dependent expression of the 3-109 reporter in L-glia. Whole-mount, third instar larval brains were stained for β-galactosidase activity (blue nuclei). mAb24B10 (brown) was used to detect photoreceptor axons. (A) Normal expression of the 3-109 reporter. (B) Expression in a partially innervated *sine oculis* mutant. Anterior is to the top, the lateral margin of the brain is to the right. Arrowheads indicate the optic stalk; open arrow, termini of retinal axons R7-8; ed, eye disc; la, lamina. Scale bar, 20 μm.

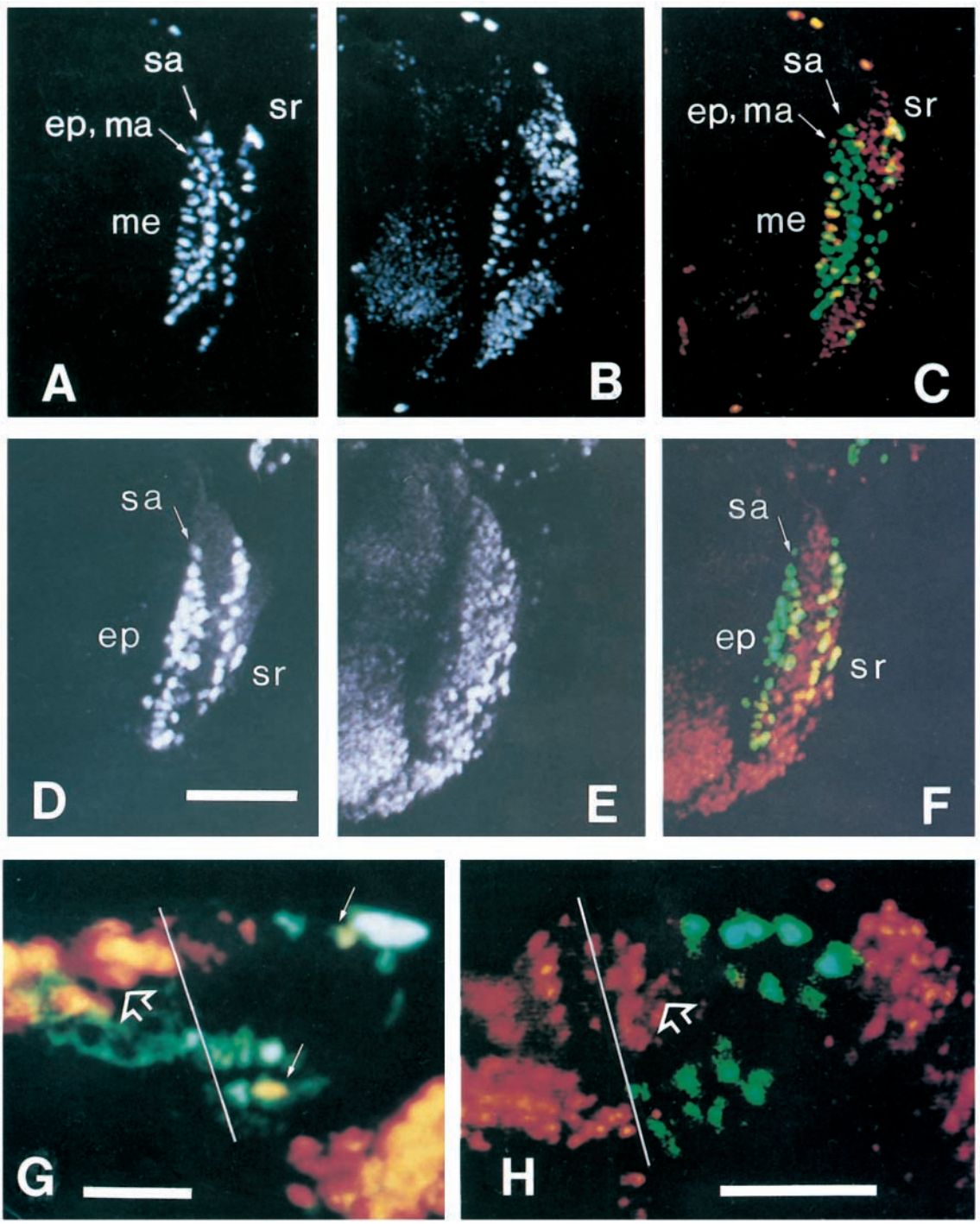

Fig. 4. BrdU labelling of L-glia in wild type. Short pulses and pulse-chases were initiated prior to innervation. Whole-mount, third instar larval brains were stained with anti-β-galactosidase antibody (panels A, D, and green/yellow nuclei in panels C, F-H) and anti-BrdU antibody (panels B, E, and red/yellow nuclei in panels C, F-H) and viewed by confocal microscopy. Panels A-C and D-F show two examples of a short pulse given in early 3IL followed by a chase to late 3IL. (A, D) Glial cells. (B, E) Cells whose precursors incorporated BrdU at the time of the pulse. (C, F) Yellow nuclei indicate co-localization of BrdU and the glial marker. (G) Short pulses during late 3IL efficiently label LPCs (open arrow) and occasionally label glial cells (closed arrow). The extensive BrdU incorporation at the lower right is in a separate proliferative domain, within the inner proliferation center (IPC, White and Kankel, 1978). (H) Short pulses during late 3IL chased to very late 3IL show a column of BrdU chasing from LPCs into the lamina (open arrow), but not into the L-glia. BrdU incorporated in the IPC is now seen in the upper right. Lines in G, H indicate the anterior margin of the developing lamina. Anterior is to the top and lateral to the right in panels A-F. Anterior is to the left and lateral to the top in G, H. Abbreviations as in Fig 1. Magnification is the same for A-F. Scale bars, 50 μm.

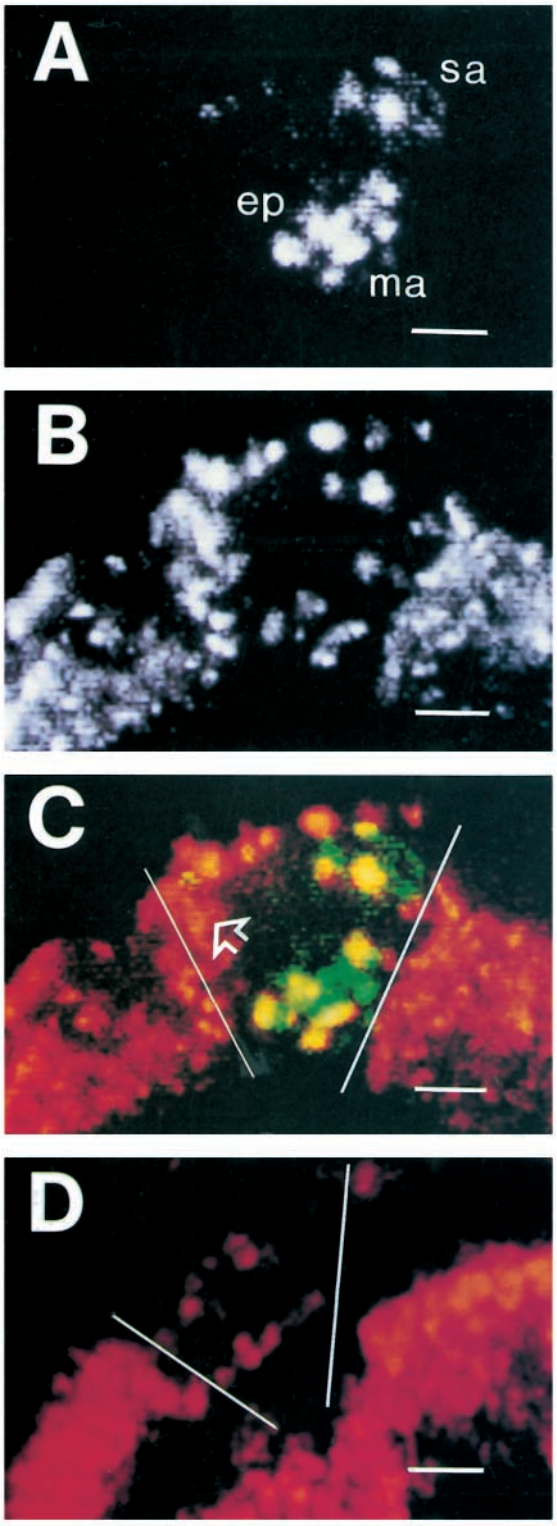

Fig. 5. Long BrdU pulses in late third instar. Whole-mount, late 3IL brains were stained with anti-β-galactosidase antibody (panel A, green/yellow nuclei in panels C, D) and anti-BrdU antibody (panel B, red/yellow nuclei in panels C, D) and viewed by confocal microscopy. Panels A-C represent a single wild-type animal. (A) Glial cells. (B) Cells which took up BrdU during the 6-hour pulse, including extensive incorporation in the proliferation zones (right and left) and restricted incorporation into the developing lamina (center). (C) Yellow indicates co-localization; many of the satellite, epithelial and marginal glial cells are BrdU-positive. Products of LPC divisions have chased into the developing lamina (open arrow). (D) A long BrdU pulse in a *sine oculis;* 3-109 animal lacking retinal innervation. No β-galactosidase was detected. Borders of the developing lamina are given by white lines in panels C, D. Anterior is to the left and lateral to the top of each panel. Abbreviations as in Fig 1. Scale bars, 20 μm.