Signals transmitted along retinal axons in *Drosophila*: Hedgehog signal reception and the cell circuitry of lamina cartridge assembly

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SUMMARY

The arrival of retinal axons in the brain of *Drosophila* triggers the assembly of glial and neuronal precursors into a ‘neurocrystalline’ array of lamina synaptic ‘cartridges’. Hedgehog, a secreted protein, is an inductive signal delivered by retinal axons for the initial steps of lamina differentiation. In the development of many tissues, Hedgehog acts in a signal relay cascade via the induction of secondary secreted factors. Here we show that lamina neuronal precursors respond directly to Hedgehog signal reception by entering S-phase, a step that is controlled by the Hedgehog-dependent transcriptional regulator Cubitus interruptus. The terminal differentiation of neuronal precursors and the migration and differentiation of glia appear to be controlled by other retinal axon-mediated signals. Thus retinal axons impose a program of developmental events on their postsynaptic field utilizing distinct signals for different precursor populations.

Key words: *Drosophila*, Lamina, Photoreceptor axon, hedgehog, Cubitus interruptus

INTRODUCTION

The survival and differentiation of postsynaptic cell populations often depends on the arrival of afferent axons in their target fields. For example, arriving retinal axons are necessary for the establishment of the retinorecipient layers of the tectum (Kelly and Cowan, 1972; Yamagata et al., 1995). In *Manduca*, the establishment of glomeruli units of the olfactory bulb depends on sensory innervation (Oland and Tolbert, 1996). Thus growing axons not only detect environmental cues for guidance and target recognition, but may emit signals that elicit the survival or maturation of cells in their prospective target fields. The molecular identities of such signals have in some cases been determined. Agrin (Rupp et al., 1991; Smith et al., 1992; Tsim et al., 1992) and Aria (Falls et al., 1993), for example, are transported to motor axon termini where they mediate the assembly of the postsynaptic apparatus at the neuromuscular junction.

The visual system of *Drosophila* offers a unique opportunity to investigate these kinds of axon-target interactions. The *Drosophila* visual system is a complex and finely ordered structure composed of tens of thousands of neurons and glia (reviewed by Meinertzhagen and Hanson, 1993). It consists of the compound eyes and the optic ganglia, which are the visual processing centers of the brain. Each of the approximately 800 ommatidial units of the compound eye contains 8 photoreceptor neurons (R-cells) that project retinotopically into distinct ganglion layers of the brain. The R1-R6 photoreceptors send their axons to destinations in the lamina, where they establish synaptic connections in a ‘neurocrystalline’ field of precisely repeating ‘cartridge’ units (Braitenberg, 1967; Meinertzhagen and O’Neil, 1991; Meinertzhagen and Hanson, 1993).

In *Drosophila* and other arthropods, the development of the visual portion of the brain is tightly regulated by the ingrowth of axons from the compound eye (Power, 1943; Macagno, 1979; Fischbach and Technau, 1984; Selleck and Steller, 1991; reviewed by Kunes and Steller, 1993). As R-cell differentiation and ommatidial assembly progress in a posterior-to-anterior order across the eye disc epithelium, the arrival of retinal axons in the brain triggers cartridge neuron precursors (LPCs) to complete a final cell division and commence neural differentiation (Fig. 1; Selleck and Steller, 1991). Retinal axons also elicit the migration of glial precursors into the lamina target field, where they mature into glia of several distinct types (Fig. 1; Winberg et al., 1992; Perez and Steller, 1996). These events result in the assembly of a retinal axon fascicle and a set of neuronal and glial precursors into a stereotyped cellular ensemble known as a ‘lamina column’ (Fig. 1C,D; Meinertzhagen and Hanson, 1993). The choreography of axon fascicle, target cell interactions yields a one-to-one correspondence of ommatidial and cartridge units, and sets the stage for the subsequent establishment of precise synaptic circuitry.

One of the signals that retinal axons deliver to the lamina is the secreted product of the *hedgehog* gene (*hh*; Huang and Kunes, 1996; Nusslein-Volhard and Wieschaus, 1980; Lee et al., 1992; Mohler and Vani, 1992; Tabata et al., 1992; Tashiro et al., 1993). Hedgehog (Hh) is expressed by differentiating photoreceptor cells immediately posterior of the
morphic and morphogenetic furrow in the eye imaginal disc (Lee et al., 1992) and is a signal to more anterior cells to enter the pathway of photoreceptor cell determination (Ma et al., 1993; Heberlein et al., 1993, 1995; Dominguez and Hafen, 1997). We have recently provided evidence that Hh is transported from the eye into the brain along retinal axons and triggers the initial steps of LPC maturation (Huang and Kunes, 1996). Hedgehog activity in the eye is both necessary and sufficient for the early events of lamina neurogenesis, including the terminal cell division of LPCs and the onset of their expression of early differentiation markers. Thus Hh acts as a dual signal that couples neural development between the eye and brain.

In the development of many Drosophila tissues, Hh acts in a signal relay cascade via the induction of secondary secreted factors, such as decapentaplegic and wingless (Basler and Struhl, 1994; Capdevila and Guerrero, 1994; Tabata and Kornberg, 1994). In the lamina, Hh might be transmitted directly by retinal axons to G1-phase LPCs to trigger their cell cycle progression and subsequent differentiation. Alternatively, LPC maturation might depend all or in part on secondary signals delivered by an intermediate Hh target. To better resolve these events, we have examined the Hh-dependence of glia cell maturation and the autonomy of Hh signaling pathway components in different lamina cell populations. Here we show that LPCs respond directly to Hh signal reception by entering S-phase, and that this step is controlled by the Hh-dependent transcriptional regulator Cubitus interruptus. Glia precursors, though Hh responsive, apparently depend on some other retinal axon-mediated signal for their migration and differentiation in the lamina target field. Thus retinal axons communicate directly to lamina precursors by utilizing different signals for distinct precursor populations.

MATERIALS AND METHODS

Mosaic analysis

The appropriate crosses were set up as described below and, following egg collection, larvae were heat shocked at about 6 hours post-hatching for 70 minutes at 37°C to induce hsFLP transgene expression. The larvae were grown at 25°C (unless otherwise noted) before dissection at late third instar stage. Brain samples from these larvae were then stained with the appropriate antibodies. When misexpression of Ci was accomplished by using a P[UAS-Ci] strain background, as described above (see also Huang and Kunes, 1996). Misexpression of Ci was accomplished by using a P[UAS-Ci] line (Alexandre et al., 1996) and a ‘flip-out’ GAL4 line, P[Tub>CD2>GAL4] (Pignoni and Zipursky, 1997). This experiment was also performed in the ‘eyeless’ condition achieved by the use of the hhts2 strain background. Crosses were set up at 18°C, and larvae were shifted to 28°C after hatching to prevent R-cell formation. A 30 minute heat shock at 37°C during the early second instar stage induced the expression of the hsFLP transgene, and activated GAL4 expression in somatic clones. The dominant-negative truncated form of Ci (C766) was misexpressed using a P[UAS-C766] line (Aza-Blanc et al., 1997) and the ‘flip-out’ GAL4 driver. The corresponding crosses are listed below:

- hh: y, hsFLP122; hhts2/TM6B, Tb × yw; P[Tub>y>y>hh]+; hhts2/TM6B, Tb
- Ci: y, hsFLP122; hhts2, P[UAS-Ci]/TM6B, Tb × yw; P[Tub>CD2>GAL4]//y+y, CyO; hhts2/TM6B, Tb
- DN-Ci: y, hsFLP122; P[UAS-Ci]/TM6B, Tb × yw; P[Tub>CD2>GAL4]//y+y, CyO

Immunohistochemistry

The CNS was dissected from late third instar larvae, fixed and stained as described previously (Kunes et al., 1993). The ombP enhancer trap line (Sun et al., 1995) and Antibody (gift of G. O. Pflugfelder) were used interchangeably, with similar results. Patched gene expression was monitored with the ptc promoter-lacZ fusion line, FE3 (Alexandre et al., 1996). Anti-Repo polyclonal antibody (Halter et al., 1995), anti-OMB antibody (Grimm and Pflugfelder, 1996) and anti-Ci antibody (Motzny and Holmgren, 1995) were used at dilutions of 1:200, 1:400 and 1:20, respectively. S-phase cells were labeled by 5-bromo-2'-deoxy-uridine (BrdU) incorporation (Truman and Bate, 1988) and detected with anti-BrdU antibody (1:50, Becton-Dickinson). Conditions for other primary and secondary antibodies were described previously (Kapning and Kunes, 1994; Huang and Kunes, 1996). Confocal microscopy was performed on a Zeiss LSM 410 microscope equipped with a Krypton/Argon laser.

RESULTS

Formation of precartridge ensembles in the developing lamina field

As an ommatidial axon fascicle arrives in the lamina target field, it assembles with neuronal and glial precursors into a stereotyped precartridge ensemble known as a lamina column (Fig. 1; Meierertzhangen and Hanson, 1993). The neuronal and glial precursors have distinct origins within the optic lobe. Most neuronal precursors (LPCs) are incorporated into the lamina target field at the anterior margin of the lamina furrow (Fig. 1A,C). The precursors of several glia cell types, on the other hand, arise in anlagen adjacent to the dorsal and ventral margins of the lamina (Fig. 1A,B) and migrate into the lamina along an axis perpendicular to that of LPC entry (Perez and Steller, 1996). The neuronal and glia precursor populations are interwoven into cell-type specific layers at the anterior margin of the lamina, so that a lamina column harbors a particular neuronal or glial cell-type precursor at a specific mediolateral position (Fig. 1C,D). Lamina neurons L1-L4 form a stack in a superficial layer, while L5 neurons reside in a medial layer near the R1-R6 axon termini. Epithelial and marginal glia are located above and below the R1-R6 termini, respectively. Satellite glia are interspersed among the neurons of the L1-L4 layer.

A number of markers distinguish glial and neuronal precursor cells from the corresponding mature cell types. The expression of optomotor-blind (omb; Heisenberg, 1972; Pflugfelder et al., 1990) labels both glial precursors in the dorsal and ventral
anlagen and mature glia that have migrated into the lamina target field (Fig. 1B,D; Poeck et al., 1993). The glia cell marker Repo (Fig. 2A,D; Xiong et al., 1994; Halter et al., 1995) and the enhancer-trap lacZ insertion 3-109 (not shown; Winberg et al., 1992) are expressed by glia once they have entered the lamina target field. Cubitus interruptus (Ci), a transcriptional mediator of Hh signaling (Eaton and Kornberg, 1990; Orenic et al., 1990; Domiguez et al., 1996; Alexandre et al., 1996; Hepker et al., 1997) is expressed by LPCs anterior of the lamina furrow and by the postmitotic neuronal precursors within the lamina (see Fig. 4D,F). The nuclear protein Dachshund (Dac; Mardon et al., 1994) is expressed only by neuronal precursors that have begun terminal differentiation and lie posterior of the lamina furrow (Fig. 1B,D; Huang and Kunes, 1996). Thus, Omb and Ci label the glial and neuronal precursors, respectively, while the mature cells, following their interaction with retinal axons,
additionally express Repo and Dac. In the lamina target field of 'eyeless' mutants, such as *eyes absent* (*eya*) or *sine oculis* (*so*), Dac expression is not detected and Repo expression is greatly diminished (Fig. 3E; Perez and Steller, 1996; Huang and Kunes, 1996).

**The migration and early differentiation of lamina glia are independent of Hh**

Enhanced transcription of the putative Hh receptor, *patched* (*ptc*; Nusslein-Volhard and Wieschaus, 1980; Hooper and Scott, 1989; Nakano et al., 1989; Ingham et al., 1991; Marigo et al., 1996a; Stone et al., 1996) is a universal characteristic of Hh signal reception (Hidalgo and Ingham, 1990; Phillips et al., 1990; Capdevila et al., 1994; Tabata and Kornberg, 1994; Heberlein et al., 1995; Goodrich et al., 1996; Marigo et al., 1996b; Vortkamp et al., 1996). All classes of glia in the lamina region upregulate *ptc* expression in a *hh* dependent fashion (Fig. 2; Huang and Kunes, 1996). These cells are thus Hh-responsive. As shown in Fig. 2F, all three classes of lamina glia, as well as medulla glia, that express a *ptc-lacZ* reporter construct are in close proximity to Hh-bearing retinal axons. We have previously shown that glia cell *ptc* reporter gene expression is not observed in *hh* animals (data not shown; Huang and Kunes, 1996). This raises the question of whether Hh signal reception is responsible for the migration and/or subsequent maturation of glia cells.

To determine whether the migration of glial precursors into the lamina target field is Hh-dependent, we examined the distribution of Omb-positive cells in *hh* animals. In the wild type, a 'trail' of Omb-positive cells delineates a path of glia migration from the dorsal and ventral anlagen (arrowheads in Fig. 3A). The clonal relationship between the Omb-positive cells in the lamina and in the glial anlage was confirmed by lineage studies (S. K., unpublished observations) in which clones were marked by a 'flp-out' lacZ reporter construct (Basler and Struhl, 1994). Omb-positive cells of the lamina, the glia anlagen and the putative migration pathway were included within the same clones. In an *eya* animal, Omb-positive cells are largely absent from the lamina target field (Fig. 3B), with the exception of the medulla glia, which are distinguished by their larger size and medial location adjacent to the medulla neuropil (not shown). Omb expression in the dorsal and ventral glia anlagen is normal in *eya* animals (Fig. 3B), consistent with previous observations using other glia precursor markers and ‘eyeless’ mutant strains (e.g. *sine oculis*; Perez and Steller, 1996).

We determined whether glia precursor migration is Hh-dependent by examining the distribution of Omb-positive cells in *hh* animals. *hh* is a regulatory mutation that specifically affects Hh expression in the visual system. In *hh* animals, approximately 12 columns of ommatidia initiate differentiation in the eye imaginal disc before the anterior progression of the morphogenetic furrow ceases (Heberlein et al., 1993). *hh* retinal axons lack Hh immunoreactivity by the time they reach the lamina target field and thus the Hh-dependent steps of LPC maturation fail to occur in *hh* animals (Huang and Kunes, 1996). As shown in Fig. 3C, Omb staining reveals a relatively normal number of glia precursors in the lamina target field of *hh* animals, despite the absence of Dac induction (see also Fig. 3F). The Omb-positive cells are distributed uniformly along the dorsoventral axis among the retinal axon fascicles, but appear more closely spaced than in the wild type. A likely explanation for this spacing defect is the absence of the neuronal precursors (Huang and Kunes, 1996) that would constitute the majority of lamina cells at this point of development.

![Fig. 2. Derepression of ptc transcription in glia and neuronal precursors. Upregulation of ptc expression in the lamina, as viewed from a lateral perspective (A-C) or a horizontal perspective (D-F). The specimens are co-labeled with anti-Repo antibody to visualize glia (blue color; shown separately in A,D), anti-β-galactosidase antibody to visualize ptc-lacZ (FE3) reporter expression (red color; shown separately in B,E) and anti-HRP to visualize neuronal membranes, including retinal axons (green color; all panels).

(A-C) The focal plane is through the epithelial glial layer, though some neuronal precursor cells are present. In C, purple cells are expressing both ptc and Repo, while the β-galactosidase-positive, Repo-negative cells are red. (D-F) As seen from the horizontal perspective, the satellite (S-glia), epithelial (E-glia), marginal (Ma-glia) and medulla glia (Md-glia) express the ptc-lacZ reporter. Subretinal glia, located on the lateral surface of the lamina (see D), do not express the ptc-lacZ reporter. Cells of the neuronal precursor layer (LPCs) are also β-galactosidase-positive. Significant levels of ptc-lacZ expression are first detectable in neuronal precursors at the trough of the lamina furrow (If; see E), where G1-phase LPCs apparently receive a retinal axon-mediated signal that triggers entry into S-phase. In A-C, anterior is to the left, dorsal is up. In D-F, anterior is to the left, lateral is up. Scale bars, 10 μm in A; 5 μm in B, C; 10 μm in D-F.]
To determine whether the glial precursors that enter the lamina target field in hh−/− animals express a retinal innervation-dependent marker, we examined their expression of Repo. As noted above, Repo-positive cells are largely absent in eya and hhts2 animals (Fig. 3E). Occasionally, a small number of Repo-positive cells are observed at ventral or dorsal lamina positions (the arrowhead in Fig. 3E). In hh+/− animals, the Omb-positive cells within the lamina also express Repo (Fig. 3F). Moreover, the Repo-positive cells occupy proper layers above and below the R1-R6 axon termini expected for satellite, marginal and epithelial glia, though the lack of markers specific for these three glia types precludes an unambiguous determination of glial cell type. The presence of marginal and epithelial glia is consistent with the observation that R1-R6 growth cones terminate in their proper positions between these layers in hh−/− animals (not shown; Huang and Kunes, 1996).

The ectopic expression of Hh in the brains of ‘eyeless’ animals is sufficient to induce the initial steps of LPC maturation in the absence of retinal axons (Huang and Kunes, 1996). For example, photoreceptor differentiation can be prevented by shifting a hh−/− animal to the nonpermissive condition at an early larval timepoint. hh+/+ somatic clones generated by a ‘flp-out’ hh construct (Zecca et al., 1995) in the brains of these animals induce the LPC terminal division and the onset of Dac expression. To determine whether either Hh or the Hh-mediated events of LPC maturation are sufficient for glia cell migration and maturation, we examined such animals for the presence of Repo-positive cells in the vicinity of hh+/+ clones within the lamina target field. Ectopic hh+/+ clones in various positions throughout the lamina target region, as well as other sites within the optic lobe, failed to induce Repo expression (data not shown). Thus neither Hh nor a secondary signal provided by the maturation of LPCs is sufficient to induce glia cell maturation.

**LPCs require smoothened for cell cycle progression and neuronal differentiation**

The activities of a number of hh signal transduction pathway components are now well characterized (reviewed in Ingham, 1995; Hammarschmidt et al., 1997). Mutations at these loci have been shown to either mimic or block Hh signal reception in a cell-autonomous fashion (see below). Examining the cellular requirements for these genes in mosaic animals should help illuminate the cellular circuitry that mediates the Hh-dependent events of lamina development.

The seven-pass transmembrane protein encoded by smoothened (smo; Nusslein-Volhard et al., 1984; Alcedo et al., 1996; van den Heuvel and Ingham, 1996) acts downstream of the Hh receptor Patched as a positive effector of Hh signal reception (Chen and Struhl, 1996). If Hh exerts its effects directly on LPCs, we would expect that loss of smo function should block the entry of G1-phase LPCs into S-phase and/or prevent the expression of Hh-dependent markers of lamina differentiation such as Dac. We used the FLP/FRT system (Golic and Lindquist, 1989; Xu and Rubin, 1993) to generate mosaic animals harboring somatic clones homozygous for the

![Fig. 3.](image-url)
null allele smo\textsuperscript{3} (Nusslein-Volhard et al., 1984). Homozygous smo\textsuperscript{3} clones were visualized by the absence of an arm-lacZ marker residing on the smo\textsuperscript{3} chromosome of a smo\textsuperscript{3} animal (blue color in Fig. 4; Vincent et al., 1994). The majority of smo\textsuperscript{3} clones recovered included the region anterior or immediately posterior of the lamina furrow. smo\textsuperscript{3} clones localized to the anterior of the lamina furrow often appeared to slow or block its anterior progression, as indicated by relative posterior position of the lamina furrow in the vicinity of such clones (Fig. 4A). Clones spanning the lamina furrow were particularly informative, as smo cells posterior of the furrow (i.e., within the lamina) were in all cases Dac-negative (Fig. 4A,C). In all such cases examined, smo\textsuperscript{+} cells adjacent to these clones within the lamina were Dac-positive. Thus, with respect to lamina differentiation, smo acts cell autonomously. smo\textsuperscript{+} clones that extended to the posterior of the lamina were rare. It is possible that LPCs that cannot respond to Hh are not readily incorporated into the lamina and displaced by smo\textsuperscript{+} LPCs. LPCs that are unable to respond to Hh might be eliminated by cell death, as occurs in ‘eyeless’ mutant animals (Selleck et al., 1992).

A hallmark of Hh signal reception in many Drosophila tissues is an increase in immunoreactivity to the C-terminal portion of the protein Ci, a transcriptional mediator of Hh signaling (Motzny and Holmgren, 1995; Johnson et al., 1995; Slusarski et al., 1995). This enhanced Ci immunoreactivity is due to inhibition of Ci proteolytic processing, a cellular response to Hh signal reception (Aza-Blanc et al., 1997). LPCs posterior of the lamina furrow display the enhanced Ci immunoreactivity that would be predicted for Hh signal reception by LPCs (see Fig. 4D,F). In animals in which hh\textsuperscript{−/−} retinal axons innervate the lamina target field, cells posterior to the lamina furrow display a level of Ci immunoreactivity equivalent to the basal level detected anterior to the furrow (not shown), indicating that the increased Ci observed in the wild type is Hh-dependent. In smo mosaic animals, smo\textsuperscript{+} cells either anterior or posterior of the lamina furrow display a basal level of Ci immunoreactivity, (Fig. 4F) while smo\textsuperscript{−/−} cells immediately adjacent to the portion of smo clones within the lamina display the high Hh-dependent level.

The initial response of LPCs to the arrival of Hh-bearing retinal axons would appear to be entry into S-phase at the lamina furrow. To determine whether cell cycle progression is directly dependent on Hh signal reception, we examined the incorporation of bromodeoxyuridine (BrdU) into S-phase cells in smo mosaic animals. In the wild type, LPCs that have entered their terminal S-phase form a discrete and continuous band at the posterior margin of the lamina furrow (Fig. 5A). In ‘eyeless’ animals lacking photoreceptor innervation or animals in which photoreceptor axons lacking functional Hh enter the lamina target field, only a low background of scattered S-phase cells are detected (see Fig. 7H; Selleck and Steller, 1991).
unclear whether the products of these scattered divisions are incorporated into the lamina (i.e., that these cells are indeed LPCs; see Selleck et al., 1992, for further discussion of these cells). In smo3 mosaic animals, mutant clones that included the posterior margin of the lamina furrow lacked S-phase LPCs (Fig. 5B). In contrast, the scattered S-phase cells anterior to the lamina furrow, and the distribution of S-phase cells in other proliferation centers, such as the OPC, were unaffected by the loss of smo function. At the lamina furrow, smo+ cells bordering smo clones were often found in S-phase. Thus, in sum, smo+ behaved as a cell-autonomous requirement for LPCs to initiate the Hh-dependent steps of lamina differentiation.

**Activation of the hh signaling pathway results in cell-autonomous LPC maturation**

The Hh receptor Ptc, a multiple-pass membrane protein, and the cAMP-dependent protein kinase (PKA) normally maintain the Hh signal transduction pathway in a repressed state (reviewed in Ingham, 1995; Hammerschmidt et al., 1997). Loss-of-function mutations in either of these genes mimic Hh signal reception and result in the cell autonomous activation of Hh target genes in many tissues (Ingham et al., 1991; Jiang and Struhl, 1995; Lepage et al., 1995; Li et al., 1995; Pan and Rubin, 1995; Strutt et al., 1995). If LPC differentiation is indeed induced directly by Hh, LPCs harboring mutations for either pka or ptc should undergo differentiation cell-autonomously and independently of retinal innervation. To test this prediction, we generated somatic clones for pka or ptc in a hhts2 mutant background, where an early shift to the nonpermissive temperature was used to block photoreceptor cell differentiation. Clones homozygous for the pka allele DC0h2 or the ptc allele, ptc0p, behaved similarly. In both cases, cells within homozygous clones in the lamina region expressed lamina differentiation markers despite the absence of retinal axons, while neighboring pka+ or ptc+ cells did not (Fig. 6A-C and 6D-F, respectively). The marked clones often spanned a region including the OPC and the LPCs, containing populations both anterior and posterior of the lamina furrow. In these clones, cell-autonomous induction of the lamina differentiation marker Dac was observed exclusively in mutant cells posterior of the lamina furrow. Mutant cells anterior of the furrow did not differentiate precociously. This observation is consistent with the consequences of ectopic Hh expression in an ‘eyeless’ mutant background (Huang and Kunes, 1996). Hh expression in regions anterior of the lamina furrow did not induce precocious lamina differentiation, as though ‘competence’ to respond to Hh is acquired by G1-phase LPCs at the anterior margin of the lamina furrow. Within the lamina target field, wild-type cells neighboring the DC0h2 or ptc0p mutant cells were never observed to express Dac. Thus activation of the Hh pathway by loss-of-function in either gene results in a strictly autonomous induction of LPC maturation. These results permit the conclusion that the terminal cell division and differentiation of LPCs both require the direct reception of the Hh signal.

**LPC cell cycle progression and cell fate determination are jointly controlled by the transcriptional regulator, Cubitus interruptus**

In a number of instances, pattern formation mediated by Hh is accompanied by cell division. The well-defined pattern of Hh-induced cell division in the lamina provides an opportunity to determine the point at which the Hh signal reception engages the cell cycle machinery. Biochemical and epistasis experiments have placed the zinc finger molecule Ci downstream of all other hh signaling pathway components (Motzny and Holmgren, 1995; Sanchez-Herrero et al., 1996; Robbins et al., 1997; Sisson et al., 1997; Chen et al., 1998). Ci has been shown to bind directly to the regulatory sequences of Hh-responsive genes (Alexandre et al., 1996; Von Ohlen et al., 1997; Von Ohlen and Hoover, 1997; Aza-Blanc et al., 1997). Should all Hh-mediated events of LPC maturation be found to depend on Ci function, we could conclude that, at least with regard to cell proliferation and the expression of differentiation markers, there is no ‘branchpoint’ within the signaling pathway.

To examine the requirement for Ci, we utilized two recombinant constructs that result in either dominant Ci gain-of-function or loss-of-function phenotypes. Overexpression of

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**Fig. 5. smo is required cell-autonomously for LPCs to enter S-phase.** Cells in S-phase were identified by incubating whole-mount tissues immediately after dissection in culture medium containing BrdU, as described in Materials and Methods. (A) In the wild type, LPCs in their terminal S-phase are visualized as a discrete band (red color; anti-BrdU antibody staining) that runs along the posterior margin of the lamina furrow (lf). Retinal axons and other neuronal membranes are visualized by anti-HRP antibody staining (green color). S-phase cells of the outer proliferation center (OPC) and of the inner proliferation center (adjacent to the lobula, lob) are also visible to the anterior and posterior, respectively, of the lamina. (B–B′) In a smo3 mosaic specimen, homozygous smo3 cells are marked by the absence of arm-lacZ reporter gene expression (blue color in B′ and B″; see the legend to Fig. 4 for details). In smo2 clones that span the lamina furrow, LPCs are not detected in S-phase at the posterior margin of the lamina furrow (red color in B and B″), in contrast to smo+/smo+ cells immediately bordering the mutant clones. Other cell proliferation centers, such as the OPC, are not affected by the loss of smo function. In all panels, anterior is to the left, dorsal is up. Scale bar, 20 μm in A for all panels.
the wild-type Ci gene results in a gain-of-function phenotype that mimics activation of the Hh signaling pathway (Alexandre et al., 1996; Domiguez et al., 1996; Hepker et al., 1997). Expression of an amino terminal fragment of Ci (hereafter referred to as DN-Ci) results in a dominant loss-of-function phenotype, as the normal in vivo function of this portion of the molecule appears to be transcriptional repression of Hh target genes (Aza-Blanc et al., 1997; Hepker et al., 1997). Both of these constructs employ the yeast UAS promoter, so that they can be expressed in somatic clones of GAL4-expressing cells (Brand and Perrimon, 1993) generated by a ‘flip-out’ GAL4 construct (Pignoni and Zipursky, 1997).

With either construct, ectopic expression in the lamina region resulted in the expected phenotype with respect to the lamina differentiation marker Dac. Dac expression in cells posterior of the lamina furrow was strongly reduced or undetectable in cells expressing DN-Ci (Fig. 7B). Conversely, the ectopic expression of wild-type Ci resulted in the induction of Dac-positive cells in the lamina target field of ‘eyeless’ animals (Fig. 7I). The effects observed with either construct were strictly cell autonomous (Fig. 7B,C; data not shown). Thus our results with ectopic Ci and DN-Ci expression are consistent with the expectation that Ci modulates Hh signaling activity directly in LPCs.

To determine whether Hh signaling acts via Ci to regulate the G1- to S-phase transition of LPCs at the lamina furrow, we examined the incorporation of BrdU into S-phase cells in animals harboring clones expressing either of the two constructs described above. As shown in Fig. 7E, cells expressing DN-Ci at the posterior margin of the lamina furrow failed to enter S-phase. Where clones of DN-Ci-expressing cells traversed the lamina furrow, S-phase LPCs are absent (Fig. 7E), while S-phase LPCs are observed immediately outside of the clone (arrowhead in Fig. 7E). Moreover, the effect on cell division was limited to the LPCs at the lamina furrow. No defects were observed in other proliferation zones such as the OPC or IPC, the other major proliferation centers of the optic lobe when they contained DN-Ci-expressing cells. Conversely, the induction of lamina differentiation by ectopic Ci expression in a hh

**DISCUSSION**

The assembly of a lamina cartridge, an intricate structure composed of five lamina neurons and six photoreceptor cell axons (reviewed in Meinertzhagen and Hanson, 1993), can be viewed as occurring in two distinct stages. Initially, an arriving ommatidial axon fascicle elicits the formation of a precartridge cellular ensemble (a lamina column; Fig. 1C) by triggering the terminal differentiation of both glial and neuronal precursors. The activity of each ommatidial fascicle in establishing a cartridge ensemble results in a one-to-one correspondence of ommatidial and cartridge units. The second stage of cartridge formation occurs later when the six R1-R6 axons of an ommatidial fascicle separate, migrate laterally and form their synaptic connections with the neurons of six neighboring cartridges (Trujillo-Cenoz and Melamed, 1973). In the adult lamina a cartridge thus receives its complement of R1-R6 axons from six different ommatidial units, none of which were members of the fascicle that originally established the cartridge cell ensemble. The mechanism by which this remarkable feat of ‘axon-shuffling’ occurs is unknown.

Here we have focused on the signaling events by which an ommatidial axon fascicle elicits the assembly of a cartridge ensemble. It is clear that this process involves specific communication events between the axons and neuronal and glial precursors. Retinal axon-mediated signals trigger LPC maturation (Selleck and Steller, 1991; Huang and Kunes, 1996), the specification of L1-L5 neurons (Huang and Kunes, 1996), and the migration and subsequent maturation of glia in the lamina target field (Winberg et al., 1992; Perez and Steller, 1996).
Here we have addressed the question of whether these events involve the same or different retinal-axon mediated signals, and whether these signals act directly or via the induction of secondary signals from resident cell populations.

Hedgehog is one of the signals that retinal axons deliver to the lamina (Huang and Kunes, 1996). Hh immunoreactivity is found on retinal axons at the time of their ingrowth into the lamina. Hh is required for lamina differentiation (see Fig. 3, for example) and is sufficient for the onset of LPC maturation when expressed ectopically in the brain. Nonetheless, in many tissues Hh exerts at least some of its effects through the induction or maintenance of secondary signals. In the embryo, for example, Hh maintains wingless expression, thus exerting an indirect effect on anterior compartment pattern (Hidalgo and Ingham, 1990; Ingham and Hidalgo, 1993). In the wing imaginal disc, Hh-mediated activation of dpp expression along the AP compartment border yields a morphogen gradient with activity in both compartments (Lecuit et al., 1996; Nellen et al., 1996; Singer et al., 1997). Finally, in the eye, ommatidial development can take place in cells lacking smoothened function, suggesting that the involvement of Hh is indirect (Strutt and Mlodzik, 1997). This raises the question of whether Hh is a direct signal for cartridge neuron differentiation or acts via the induction of a secondary signal, perhaps in some other cell population.

Our data indicate that neuronal precursor (LPC) differentiation in the lamina is due to direct Hh signal reception. A mosaic analysis with four Hh signaling pathway components (smoothened, patched, Protein Kinase-A and cubitus interruptus) revealed in all cases a cell-autonomous requirement for Hh signal reception in LPCs. Since lamina neurons and glia derive from distinct lineages (Winberg et al. 1993; Perez and Steller, 1996), these observations rule out a requirement for Hh signal reception in glia for LPC maturation. The requirement for smoothened for LPCs to enter S-phase and express lamina differentiation markers was observed in relatively small somatic clones that coincided with the location of G1-phase LPCs (Figs 4, 5). Wild-type LPCs immediately adjacent to those smo cells commenced cell division and differentiation.

![Fig. 7. Cubitus interruptus (Ci) regulates both LPC cell division and differentiation.](image-url)
lamina differentiation normally. Conversely, in the absence of retinal axons, activation of the Hh signaling pathway via loss of function in patched or Protein Kinase-A triggered LPC maturation in a cell-autonomous fashion (Fig. 6). These somatic clones did not induce differentiative events in adjacent wild-type LPCs, which failed to undergo differentiation due to the absence of retinal axons. This observations argue strongly against the notion that Hh signal reception triggers the expression of a secondary diffusible signal that is sufficient for LPC maturation.

Hh signal reception is in a number of instances accompanied by cell proliferation. In the chick, Sonic Hedgehog triggers cell proliferation in conjunction with its patterning activity in the somitic mesoderm (Fan et al., 1995). In the mouse, loss of patched function is associated with enhanced cell proliferation in the neural tube and a higher incidence of a proliferative disease, medulloblastoma (Goodrich et al., 1997). In Drosophila, morphogenesis induced by Hh in the developing eye includes two waves of cell division, one anterior and one posterior of the morphogenetic furrow (Ready et al., 1976; Heberlein et al., 1995). Our data indicate that, at least in the case of the lamina, Hh acts via the transcription factor Ci in regulating cell cycle progression (Fig. 7). Both genetic epistasis and biochemical experiments place Ci at a terminal step in Hh signal transduction, as a nuclear effector of the upstream components Ptc, Smo, PKA, and Fused (Motzny and Holmgren, 1995; Sanchez-Herrero et al., 1996; Robbins et al., 1997; Sisson et al., 1997; Chen et al., 1998). Thus we conclude that, at least with regard to cell proliferation and the expression of differentiation markers, there is no ‘branchpoint’ within the signaling pathway. Rather, cell proliferation would appear to be controlled by a transcriptional target of Hh. This might be a component of the cell cycle regulatory machinery, but less direct avenues are also possible.

There is evidence for the activity of at least two additional retinal axon-mediated signals in lamina cartridge assembly. First, retinal axons appear to provide at least one signal downstream of Hh that mediates the final differentiation of cartridge neurons (Huang and Kunes, 1996). Only a subset of the postmitotic LPCs generated by Hh-dependent events become cartridge neurons. Five postmitotic LPCs associated with each retinal axon fascicle begin to express neuronal markers (Fig. 1C); the remaining LPCs are eliminated by apoptosis. Hh is not sufficient for the terminal differentiation of cartridge neurons. Our recent observations (Z. H., unpublished data) indicate that a second retinal axon-mediated signal is required and that it acts via the Drosophila EGF receptor. A third retinal axon-mediated signal is apparently required for lamina glial differentiation. Though responsive to retinal axon-borne Hh (Fig. 2), the satellite, marginal and epithelial glia depend on retinal axons, but not Hh, in order to migrate into the lamina target field and undergo subsequent differentiation (Fig. 3). Because the Hh− retinal axons that can promote these events cannot trigger LPC maturation, we suppose that this third signal is independent of the Hh-mediated events of LPC maturation (Fig. 3). These events are also independent of EGF receptor activity (our unpublished observations). Thus we suppose that at least three retinal axon-mediated signals orchestrate the assembly of a lamina cartridge.

The transmission of signals from the eye to the brain is a mechanism by which the precision of ommatidial assembly in the eye is imposed on the developing postsynaptic field. As in the case of the eye (Ready et al., 1976), the assembly of a cartridge cellular ensemble proceeds according a stereotyped choreography of cellular events (Meinertzhagen and Hanson, 1993). At the anterior of the lamina, a retinal axon fascicle is joined first by a cell destined to become the L1 neuron. L-neurons appear to be added to the fascicle-associated ensemble in a cell-type-dependent sequence (Meinertzhagen and Hanson, 1993; S. Kunes, unpublished observations). This process yields a precise geometric array of retinal axon, target cell associations that set the stage for the remarkable events of synaptic cartridge formation that follow. One might suppose then that to some extent the presynaptic axons serve as a ‘template’ for the establishment of order in the lamina. Conversely, it is clear that target field cells play some role in the establishment of precise connectivity, for example in the initial guidance of photoreceptor axons (see, for example, Martin et al., 1995) and the maintenance of growth cone, target cell associations via the synthesis of nitric oxide (Gibbs and Truman, 1998). Thus, in the Drosophila visual system, there is a complex interplay between pre- and postsynaptic cell populations, many aspects of which are as yet unresolved.

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REFERENCES
Dominguez, M., Brunner, M., Hafen, E. and Basler, K. (1996). Sending and


