Target Field Response to Retinal Innervation in *Drosophila melanogaster*

by

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B.A., Wellesley College, 1989

Submitted to the Department of Biology in Partial Fulfillment of the Requirements for the degree of

Doctor of Philosophy
in Biology

at the

Massachusetts Institute of Technology
February, 1996

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ABSTRACT

This work focuses on the development of the *Drosophila* first optic ganglion, which is also called the lamina. The lamina is one of the photoreceptor neuron target fields, and contact between the eye and the brain is required for its integrity. Mutations which act in the eye to disrupt retinal innervation of the brain also disrupt the lamina. This phenotype is, in part, due to an inductive interaction between retinal axons and the developing brain. Photoreceptors trigger the final divisions of their synaptic partners, the lamina neurons (L-neurons).

An investigation was carried out to determine how retinal innervation affects the development of the lamina glial cells (L-glia). L-glia are associated with both the retinal axon termini and with the developing layer of L-neuron cell bodies. The L-neurons and L-glia arise from distinct precursor populations, which are spatially segregated within the developing brain. Lamina gliogenesis occurs independently of retinal cues, and immature glia begin to migrate into the lamina anlage prior to input from the eye. If the brain receives retinal input, glial migration continues. Rows of L-glia appear to move into the lamina just ahead of each row of retinal axons. Once these immature glia have been incorporated into the ganglion, they begin to express glial differentiation markers. However, in the absence of input from the eye, glial migration stalls. Glia begin to express early developmental markers, but require retinal cues for their terminal differentiation.

In the interest of understanding the molecular basis of communication between ingrowing photoreceptor axons and the lamina progenitors, P element enhancer trap lines were screened for those which showed reporter expression in the developing target field. A new gene, *lamina ancestor* (*lama*), was identified. Despite their distinct lineages and differential response to retinal input, both the L-glial and L-neuron precursors express *lama*. The L-glial progenitors begin to express the gene quite early in the animals life, approximately two days before they migrate into the target field. In contrast, expression in the L-neuron precursors is coincident with input from the eye. Expression in the neuronal precursors does not, however, rely on a retinal cues. In non-innervated brains, both classes of precursors express *lama*.

Null mutations in *lama* were generated, and homozygous mutants are viable and fertile. Furthermore, the visual system appears to develop normally in these mutants. Widespread, ectopic expression of *lama* is similarly innocuous. Nevertheless, it is possible that *lama* plays a role in the development of the lamina. The *D. melanogaster* protein is 74% identical to its *D. virilis* homologue, indicating that the locus has been under selective pressure over the 60 million years that separate the two fly species.

Acknowledgements

I am grateful to:
My advisor, Hermann Steller. I learned quite a lot from him. I know few scientists who are as generous as Hermann is, and few who are as persuasive.

The members of my thesis committee: Terry Orr-Weaver, Frank Solomon, Hazel Sive and Norbert Perrimon. I highly appreciate the time they've spent thinking about my work.

Members of the Steller lab and the 4th floor of E25. In particular: Megan Grether, for her sound advice,sympathetic ear, and willingness to exchange reading material; Meg Winberg, for freely offering her opinions of science and non-science related matters. I am especially grateful for being rescued from that umbrella wielding wierdo in Verona; Sam Kunes, for stimulating discussions regarding lamina formation; John Abrams, for his buffering capacity and for his good natured ribbing; Cathy Shea, for her calm nature and sense of humor; Kevin Lee and Kristin White, for always asking the relevant questions; Florence Davidson, for providing daily reality checks, sharing communication skills, and for the funniest stories I've heard in six and a half years. I am particularly fond of the one involving toe shoes; Anne-Francoise Lamblin, for never laughing at any of my molecular biology questions, going to see all those movies with me, and for letting me tease her mercilessly; and to all those other folks who made my MIT career both intellectually and socially rewarding: Eleonora Fusco, Chip Quinn, Dave Smith, Elisabeth Folkers, Asli Kusambar, David Litwack, Daniel Emerling, John Ivens, Patrick McCaw, Jae Kim, Cerinda Carboy-Newcomb, Courtney Hunter, Alba Cid, Julie Agapite, Chia Lin Wei and Jan Tittle.

Steven Perez and Mike Perez, who are two of the most talented and unaffected people that I know. They can also be counted on for the "unvarnished truth" of any situation.

Kevin Roberg, and my parents, Kathy and Jerry Perez, for their encouragement and support .

Rosie and Beeze, for their devotion. Its either to me, or to the can opener.

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I. Introduction

One of the key questions examined by developmental neurobiologists is that of how neurogenesis is controlled. A related issue is that of how diverse cell types are generated in the appropriate locations. Both processes respond to cell intrinsic programs, as well as local, extracellular cues. An additional layer of complexity is added when one considers that cells within the nervous system communicate over considerable distances. Afferent inputs have been demonstrated to influence both the proliferation and differentiation of cells within a target field (see Gong and Shipley, 1995; Currie and Bate, 1995) .

I have focused on the formation of the first optic ganglion, or lamina, in *Drosophila melanogaster*. Although the ganglion is relatively simple in structure, the mechanisms by which it develops are used throughout both vertebrate and invertebrate nervous systems.

In this chapter, I will review some of the issues which are pertinent to the development of the lamina, and describe the structure of the developing adult *Drosophila* visual system.

Generation and specification of neurons and glia

Drosophila neurogenesis

The *Drosophila* central nervous system (CNS) arises from the ectoderm during embryogenesis (Poulson, 1950; Hartenstein and Campos-Ortega, 1984). The ventral nerve cord (VNC) and the sub-oesophageal ganglion are generated by the neurogenic region of the germ band, while the brain hemispheres arise from the procephalic neurogenic region (Fig. 1A). The ventral midline is derived from a distinct subset of the neuroectoderm, which is referred to as the "mesectoderm" (Fig. 1B) (Poulson, 1950; Klämbt, et. al., 1991). Dorsolateral ectoderm gives rise to the larval peripheral nervous system (Bodmer, et. al., 1989). Early development of the insect nervous system is notably different from that of vertebrates' in two respects. Mesodermal tissue does not induce the neuroectoderm (Rao, et. al., 1991; and see Torrence et. al., 1989), and most neural precursors do not migrate far from their site of origin (but see Jacobs et. al., 1989; Copenhaver and Taghert, 1990).

Neuroblasts (NBs) delaminate from the neurogenic regions within a 5 hour period. "Delamination" refers to the segregation of NBs from the ectoderm. In the procephalic region, segregation is continuous, while, in the germ band, it occurs in five distinct waves (Hartenstein and Campos-Ortega,

1984; Doe, 1992). NBs divisions begin after delamination and are largely asynchronous, although exceptions are seen in the procephalic region. NBs will give rise to another NB and to a ganglion mother cell (GMC), which, in turn, generates two neurons, or, a neuron and a glial cell (Fig. 1C) (Doe and Goodman, 1985a; Udolph, et. al., 1993). Concurrent with NB delamination, midline precursors (MPs) segregate from the mesectoderm and sensory organ precursors (SOPs) divide within the dorsolateral ectoderm (Bodmer et. al., 1989; Klämbt et. al., 1991). Throughout the nervous system, the pattern of a neural precursor's divisions will vary according to its lineage and position (Thomas et. al., 1984; Doe and Goodman, 1985a; Bodmer et. al., 1989; Klämbt et. al., 1991; Doe, 1992; Bossing and Technau, 1994).

Following metamorphosis, the fly possesses a remodeled CNS, and a new set of peripheral sense organs which are generated by the imaginal discs. Imaginal disc progenitors are segregated from their neighbors during embryogenesis, and undergo most of their divisions during larval life (reviewed in Russell, 1982). Approximately 200 NBs within the CNS persist post-embryonically, and continue to proliferate during larva life (White and Kankel, 1978; Truman and Bate, 1988; Hofbauer and Campos-Ortega, 1990; Prokop and Technau, 1991; Ito and Hotta, 1992). A subset of these NBs become quiescent during embryogenesis, and resume proliferation in the first or second instar. The reorganization of the nervous system during larval and pupal life includes innervation of the CNS by the imaginal sense organs, modification of the CNS to accommodate new peripheral input, and the histolysis of larval sensory organs and their CNS targets (reviewed in Kankel et. al., 1980; Levine et. al., 1995).

Neurogenesis is controlled by two sets of genes: the proneural, and the neurogenic loci. Proneural genes confer neural competence on a cluster of cells. The neurogenic genes will then restrict the number of competent cells within a proneural cluster which go on to assume a neural fate (reviewed in Jan and Jan, 1994; Campos-Ortega, 1994).

The best characterized proneural genes are those of the *achaete-scute* complex (AS-C): *achaete* (*ac*), *scute* (*sc*), *lethal of scute* (*l'sc*), and *asense* (*ase*). In mutants which lack the entire AS-C, approximately 25% of NBs in the ventral neurogenic region are missing (Jimenez and Campos-Ortega, 1990).

The defect in AS-C mutants is most likely due to the absence of the *l'sc* transcript (Jimenez and Campos-Ortega, 1987). An additional 25% of NBs are specified by the *ventral nervous system condensation defective* (*vnd*) locus (Jimenez and Campos-Ortega, J. A., 1990). As 50% of NBs develop normally in AS-C, *vnd* double mutants, it is likely that additional neurogenic loci function in the developing CNS. Both *l'sc* and *vnd* are more widely expressed in the developing CNS than is evident by their phenotypes, which suggests that "neural competence" is encoded by genes with partially redundant functions (Martin-Bermudo, et. al., 1991; Jimenez, et. al., 1995). The progenitors of distinct subsets of larval and adult sensory organs are determined by *ac* and *sc*, as well as by the *atonal* (*ato*) locus (Jarman, et. al., 1993; Jarman, et. al., 1994; and reviewed in Campuzano and Modollel, 1992; Skeath and Carroll, 1994). Most of these proneural genes encode basic helix-loop-helix (bHLH) transcription factors (Campuzano and Modolell, 1992), while *vnd* encodes a homeodomain protein (Jimenez et. al., 1995).

Several mechanisms restrict the initial expression of the proneural genes to a cluster of cells. In the embryo, the expression of *ac* and *sc* are positively regulated by pattern formation genes (Skeath et. al, 1992), other proneural genes (Skeath, et. al., 1994), and by each other (Skeath and Carroll, 1991). The positions of proneural cell clusters also appear to be determined by negative regulators. In the wing disc, *extramachrochaete* (*emc*) and *hairy* (*h*) repress the expression of *ac* and *sc* outside of the proneural clusters (Skeath and Carroll, 1991; Cubas and Modolell, 1992). *emc* and *h* also repress *ato* expression in the eye disc, acting anterior to the morphogenetic furrow (Brown, et. al., 1995). Both *h* and *emc* contain HLH domains, but neither has the canonical basic domain, which is necessary for DNA binding (Rushlow, et. al., 1989; Garrell and Modolell, 1990). *h* and *emc* have been thought to negatively regulate proneural expression by heterodimerizing with, and sequestering, the ac and sc proteins. More recent data, however, indicates that *h* may also function as a repressor by directly binding DNA (Ohsako, et. al., 1994).

Intercellular communication within a proneural cluster leads to the emergence of a single neural precursor (reviewed in Jan and Jan, 1994). Proneural gene expression increases within the neural precursor, and decreases within the precursor's neighbors (Fig. 2A). Restriction of proneural

gene expression requires the neurogenic loci, which act through a process called lateral inhibition, more recently referred to as lateral specification (Artavanis-Tsakonas, et. al., 1995).

The original neurogenic mutants were identified based on a hyperplasic ventral nerve cord phenotype (Lehmann, et. al., 1983). In the absence of a neurogenic function, all cells within a proneural cluster assume a neural fate. The neurogenic loci also act to limit the total number of neural precursors in the embryonic and adult PNS (see Hartenstein and Campos-Ortega, 1986; Hartenstein and Posakony, 1990; Goriely, et. al., 1991; Rao, et. al., 1992). Among the best characterized neurogenic loci are *Notch* (*N*), *Delta* (*Dl*) and *Enhancer of split* (*E(spl)*). Two additional loci, *scabrous* (*sca*) and *Suppressor of Hairless* (*Su(H)*), restrict neural fate in subsets of the developing adult peripheral nervous system (Mlodzik, et. al., 1990; Furukawa et. al., 1992; Schweisguth and Posakony, 1992).

In order to determine whether the original neurogenic loci act in a cell autonomous manner, experiments were carried out in which single, marked cells were transplanted from the ventral neurogenic region of mutant donors into wild type hosts (Technau and Campos-Ortega, 1987). The hosts were allowed to develop, and were then examined to determine what types of cells the marked donor could generate. In a wild type background, cells homozygous for all but one of the neurogenic mutations gave rise to both neuronal and epidermal cells (but see Hoppe and Greenspan, 1986). Cells from *E(spl)* mutants overwhelmingly gave rise to neuronal cells. These data suggested that most of the neurogenic genes function to transmit an epidermal inducing signal, while *E(spl)* begins to carry out epidermal differentiation. A subsequent epistasis analysis indicated that *N* and *Dl* act at the beginning of the signal transduction pathway, while, as expected, *E(spl)* is downstream of the remainder of the loci (de la Concha, et. al., 1988).

N is a cell surface receptor and *Dl* is one of its ligands (Fehon, et. al., 1990; and reviewed in Knust, 1994) (Fig. 2b). *N* and *Dl* encode transmembrane proteins with extracellular EGF repeats (Wharton et. al., 1985; Vassin et. al., 1987). It is currently thought that all cells within a proneural cluster express equal amounts of *N* and *Dl*, until one cell attains slightly more proneural gene expression and upregulates *Dl* (reviewed in Knust, 1994). *N* is "activated" in adjacent cells by binding to *Dl*, and a signal triggering

epidermal fate is transduced to the nucleus (reviewed in Artavanis-Tsakonis et. al., 1995). Elegant molecular analyses strongly suggest that the Notch protein is able to directly transduce signals from the cell surface to the nucleus (Struhl, et. al., 1993; Lieber, et. al., 1993). When the cytoplasmic domain of Notch is artificially uncoupled from its ligand binding domain, it localizes to the nucleus and produces an "antineurogenic " phenotype. Whether or not endogenous Notch is localized to the nucleus is unclear. Notch may affect nuclear events by another mechanism, which was described by Fortini and Artavanis-Tsakonas (1994). Notch appears to sequester the Su(H) protein in the cytoplasm. When Notch binds its ligand, Delta, Su(H) is translocated to the nucleus (Fortini and Artavanis-Tsakonas, 1994). *Su(H)* encodes a DNA binding protein, and therefore has the potential to regulate transcription (Furakawa et. al., 1992; Schweisguth and Posakony, 1992). A principal target of *N* signaling is the *Enhancer of Split E(spl)* complex (Jennings et. al., 1994; reviewed in Campos-Ortega, 1994). *E(spl)* encodes seven bHLH transcription factors (see Knust, et. al., 1992), and is conserved in *Drosophila hydei* (Maier, et. al., 1993). Interestingly, none of the individual genes are mutable to lethality, suggesting that functional redundancy is at work among the neurogenic, as well as the proneural, genes.

Several of the proneural and neurogenic loci appear to regulate each other. The expression patterns of two *E(spl)* transcripts are regulated in a combinatorial manner by the *vnd* locus and the *achaete-scute complex* (Kramatscheck and Campos-Ortega, 1994). *l'sc* is sufficient to induce expression of *Dl* and of some of the *E(spl)* transcripts (Hinz, et. al., 1994). Imaginal disc expression of two of the *E(spl)* transcripts and *sca* appear to be directly regulated by *ac* and *sc* (Singson, et. al., 1994). In addition, some of the neurogenic genes may directly affect expression of proneural genes. *N* and *Dl* modulate *l'sc* expression at the level of transcription (Bermudo et. al., 1994).

Control of neuronal cell lineage in Drosophila

Fate determination within the nervous system involves both the choice between a neuronal or glial cell fate, as well as a decision regarding what type of glia or neuron will be generated. A cell's fate is affected both by a cell's lineage, and by its environment (Doe and Goodman, 1986b).

Few glial lineages in *Drosophila* have been described. Within the embryonic CNS, the origins of glia at the midline are well understood (Jacobs et. al., 1989; Klämbt et. al., 1991; Condron and Zinn, 1994). Some of these glia arise from glioblasts, while others are generated by multipotent precursors. Multipotent progenitors are also found in the ventral neurogenic region (Udolph et. al., 1993). In the embryonic PNS, all cells within the chordotonal and external sensory organs arise from a common, sensory organ precursor (SOP) (Bodmer et. al., 1989). Each organ contains one neuron, one glial cell and two outer support cells.

The genetic control of glial vs. neuronal fates is just beginning to be examined. The development of almost all embryonic glial cells, with the exception of the midline glia, requires the *glial cells missing* (*gcm*) locus (Jones, et. al., 1995; Hosoya, et. al., 1995). The *gcm* gene encodes a putative nuclear protein, and loss of function mutations are sufficient to transform glia into neurons. Over expression of *gcm* is sufficient to induce a glial fate in cells that would otherwise be neurons. The generation of some of the midline glia requires *engrailed* (*en*) function (Condron et. al., 1994). In the absence of *en*, all progeny of a particular midline precursor are neurons. In wild type animals, this precursor generates both neurons and glia. Another locus, *sandpodo* (*spdo*) is required for the formation of sensory glia (Salzberg et. al., 1994). In *spdo* mutants, sensory glia adopt a neuronal fate.

Within the peripheral sensory organs, two loci have been identified which specify the neural-glial vs. the outer support cell lineage. In *tramtrack* (*ttk*) mutants, the peripheral neurons and glia are duplicated at the expense of the outer support cells (Salzberg et. al., 1994; Guo et. al., 1995). *ttk* encodes a putative transcriptional repressor (Harrison and Travers, 1990; Brown and Wu, 1993). *numb* mutants display the converse phenotype, in which the neuron and glial cell are transformed into outer support cells (Uemura et. al., 1989). The numb protein is associated with the SOP cell membrane, and is asymmetrically distributed between daughter cells at the first division (Rhyu et. al., 1994). The loss of function phenotype of either locus is mimicked by ectopic expression of the other gene. *ttk* appears to work downstream of *numb*, and is required within the outer support cells (Guo et. al., 1995).

The specification of unique identity within the developing CNS may

involve the reactivation of some of the early embryonic pattern formation genes. Mutations in the pair rule genes eliminate alternate body segments, while mutations in the segment polarity genes disrupt the pattern within each segment (reviewed in Scott and O'Farrell, 1986).

Two of the pair rule genes, *fushi tarazu* (*ftz*) and *even skipped* (*eve*) are expressed in distinct sets of NBs (Doe et. al., 1988a; Doe et. al., 1988b). Within the midline, *ftz* is expressed concomitantly with NB delamination, while, in the bilateral neurogenic region, it is expressed in GMCs and in the lateral glioblast. When *ftz* expression is eliminated in the CNS, some of the normally ftz^+ GMCs undergo a fate transformation. In several lineages, *ftz* seems to function upstream of *eve*. Another pair rule gene, *runt*, appears to specify neural lineages in a set of NBs that partially overlap the eve^+ NBs (Duffy et. al., 1991).

Several segment polarity genes also affect the CNS, in a manner that is distinct from their affects on cuticle development (Patel, et. al., 1989). The *gooseberry* (*gsb*), *patched* (*ptc*), and *wingless* (*wg*) loci specify subsets of CNS NBs (Patel et. al., 1989). *wg* is a secreted protein (Baker, 1987), and acts non cell-autonomously within the developing CNS. wg^+ cells affect both the segregation of adjacent NBs and gene expression in neighboring cells (Chu-LaGraffe and Doe, 1993). The *gsb* locus encodes two related proteins, gsb-proximal (p) and gsb-distal (d) (Baumgartner et. al., 1987), each of which is expressed in the same NB lineages. Ectopic expression of either protein is sufficient to alter the lineage of some NBs, in a manner reciprocal to that seen in the loss of function mutants (Zhang et. al., 1994). The levels of each gsb protein vary with the developmental stage of the cell (Buenzow and Holmgren, 1995). In the NBs, gsb-d is expressed at high levels, while gsb-p is expressed at lower levels. In the progeny of the NBs, however, levels of protein expression are reversed, such that gsb-d expression is low, while gsb-p expression is high.

A number of other loci affect lineage identity in the CNS. The *polyhomeotic* locus appears to both activate *ftz* expression and repress *eve* expression in subsets of NBs (Smouse et. al., 1988). Two additional genes, dPOU28 (Dick et. al., 1991; Billin et. al., 1991) and *castor* (Mellerick et. al., 1992), were identified because of their expression pattern in the CNS. Ectopic expression of dPOU28 is sufficient to transform two, well characterized sibling

cells into their parental GMC (Yang et. al., 1993). The *castor* gene, also referred to as *ming* (Cui and Doe, 1992), encodes a zinc finger transcription factor. *castor* is expressed at restricted times during NB life, suggesting that it may act to differentially specify the NB daughter cells, and appears to be upstream of *engrailed* in some lineages.

Within the PNS, two genes have been identified which determine the type of sensory organ generated by a sensory organ precursor (SOP). The *cut* gene encodes a homeodomain protein (Blochlinger et. al., 1988), and is expressed in all cells within the external sensory (es) organs. Loss of function *cut* mutations transform (es) organs into chordotonal (ch) organs, while ectopic expression of *cut* leads to the reciprocal transformation of (ch) organs into (es) organs (Blochlinger et. al., 1991). The (es) organs have varied morphologies, and may be subdivided into "mono-innervated" vs. "poly-innervated" organs. A paired box gene, *pox-neuro* (*poxn*), is required for the development of the poly-innervated organs, and ectopic expression of *poxn* is sufficient to transform mono-innervated organs into poly-innervated organs(Dambly-Chaudiere et. al., 1992; Nottebohm et al., 1992). Recent work has indicated that *poxn* acts downstream of the AS-C, and upstream of *cut* (Vervoort et. al., 1995).

Vertebrate studies

The early development of the vertebrate nervous system has been well described morphologically. Signals from the dorsal mesoderm transform a portion of the overlying ectoderm into the neural plate (Fig. 3A; reviewed in Kessler and Melton, 1994). Subsequently, the neural plate invaginates along its anterior/posterior axis to generate the neural tube during a process called neurulation (Fig. 3B). Although some aspects of neural differentiation will occur in isolated ectodermal tissue, neurulation requires mesodermal cues (reviewed in Kessler and Melton, 1994). A migratory population of cells, called the neural crest, emerges from the dorsal aspect of the neural tube (Fig. 3C). Neural crest cells give rise to the peripheral nervous system (PNS) (reviewed in Le Douarin and Smith, 1988). Along the lumen of the neural tube, or ventricular zone, cell proliferation both increases overall cell number in the central nervous system (CNS), and generates the regional size differences, which precede the formation of the brain (reviewed in Purves

and Lichtman, 1985; Martin and Jessell, 1991).

The study of vertebrate neurogenesis at a cellular level has been aided by the ability to examine neuronal development *in vitro*. Many cell types have been successfully cultured and analyzed over limited time periods (see for e.g.. Raff et. al., 1985; Kalcheim et. al., 1992; DeHamer et. al., 1994). Of particular interest are stem cells, which are defined as self-renewing precursors with the capacity to generate daughters of a variety of lineages (reviewed in Gage et. al., 1995). Transplantation and cell marking techniques have also been useful, allowing lineage analyses to be carried out *in vivo* in a variety of tissues (see, for e.g.., Le Lievre et. al., 1980; Turner and Cepko, 1987; Wetts and Fraser, 1988). Furthermore, the isolation of counterparts for *Drosophila* genes has begun to facilitate an understanding of the molecular basis of vertebrate neurogenesis (reviewed in Calof, 1995).

Proliferation within the nervous system is controlled by a diverse set of cell types and molecules. Retinal amacrine neurons appear to inhibit the divisions of their precursors (Reh, 1987). Homotypic interactions induce the divisions of cerebellar granule neuron progenitors (Gao et. al., 1991). One class of optic nerve glial cells, the type I astrocyte, stimulates the divisions of the precursors of another class of glia, the oligodendrocyte (Raff et. al., 1985; Temple and Raff, 1986; reviewed in Raff, 1989 and Barres and Raff, 1994). A cocktail of growth factors is sufficient to replace the mitogenic signal derived from astrocytes. Two of these growth factors, Neurotrophin 3 (NT-3) and Insulin-like growth factor 1 (IGF-1), also trigger the proliferation of PNS precursors. IGF-1 is expressed in the developing sympathetic ganglia, and, will, *in vitro*, stimulate the divisions of immature sympathetic neurons (Zackenfels et. al., 1995). NT-3 increases the proliferation of migrating neural crest cells (Kalcheim et. al., 1992). Another growth factor, basic fibroblast growth factor (FGF2), stimulates the proliferation of two distinct precursors of olfactory neurons (DeHamer et. al., 1994), as well as the proliferation of striatal precursors (Vescovi et. al., 1993). FGF2 and nerve growth factor (NGF) appear to act synergistically on striatal precursors (Cattaneo and McKay, 1990). FGF1, FGF2, epidermal growth factor (EGF) and transforming growth factor α (TGFα) act as mitogens for retinal precursors, while transforming growth factor β (TGFβ) appears to halt proliferation (reviewed in Lillien, 1994).

Withdrawal from the cell cycle is not an obligatory prerequisite for differentiation. Neurons in the sympathetic ganglia (Rohrer and Thoenen, 1987) and type I astrocytes in the optic nerve (reviewed in Raff, 1989) continue to proliferate after expressing cell type specific markers.

In vivo lineage analyses have indicated that, as in *Drosophila*, both multipotent progenitors and precursors with restricted fates exist in the developing vertebrate nervous system. In these studies, dividing cells were labeled with either a replication incompetent retrovirus bearing a reporter gene (Price et. al., 1987; Galileo et. al., 1990) or by injection of impermeable, flourescently tagged molecules (Wetts and Fraser, 1987; Bronner-Fraser and Fraser, 1989). Precursors in the cerebral cortex and the postnatal olfactory bulb appear to be restricted to either a glial or a neuronal fate (reviewed in Luskin, 1994; but, see below). In contrast, in the chick optic tectum, approximately 1/3 of the precursors generate both neurons and glia, while 2/3 of the precursors generate only a single cell type (Galileo et. al., 1990). Retinal progenitors in rats are capable of generating neurons and glia throughout the period of retinal proliferation (Turner and Cepko, 1987; Turner et. al., 1990). Pluripotent cells have also been found within the neural crest, and appear to become restricted to particular lineages during the course of their migration from the neural tube (Bronner-Fraser and Fraser, 1989; and reviewed in Le Douarin and Dupin, 1992). When crest cells are marked prior to migration, nearly half the population appears pluripotent (Frank and Sanes, 1991). Following migration, up to 98% of precursors within a developing ganglion may be restricted to either a neuronal or a glial fate (Hall and Landis, 1991).

Transplantation studies have supported the notion that developmental potential may change over time. Proliferating cells within the postnatal cerebellum give rise exclusively to granule neurons (Gao and Hatten, 1994). However, when donor cells are taken from the cerebellum of younger animals (E13) and transplanted into postnatal hosts, they give rise to multiple cell types. This indicates that cerebellar progenitors lose potency over time. Transplantation of neural crest cells has indicated that they also become restricted to particular lineages (e.g.. sympathetic vs. sensory) and cell types (e.g.. neuronal vs. glial) over time (reviewed in Anderson, 1989; Douarin and Dupin, 1994). Temporal regulation of fate is also seen in the cerebral cortex and in the retina. During cortical development, the birthdate of a cell

correlates with its neuronal phenotype (reviewed in McConnell, 1992). Transplanted cortical neuronal precursors assume the phenotype appropriate for the age of their host, unless they are transplanted during G2 of their cell cycle (McConnell and Kaznowski, 1991). By G2, cortical precursors appear to have acquired the information which determines their fate, and are resistant to external signals. A correlation between birthdate and phenotype is also seen in the developing retina (reviewed in Lillien, 1994). Taking into account the results of lineage analyses (see above), it appears that a retinal progenitor's fate is determined after its final division, in response to environmental cues (reviewed in Lillien et. al., 1994).

Multipotent precursors seem to be found if they are searched for early enough and, in some tissues, will persist until late developmental stages. Environmental cues which influence a cell's fate choice appear to be temporally regulated, as does a cell's ability to respond to those cues.

The experimental manipulations of stem cells has shed light on the cellular and molecular mechanisms which control fate choices. Neural crest stem cells express the tyrosine kinase receptor, c-Neu (Stemple and Anderson, 1989; Shah et. al., 1994). In response to either glial growth factor (GGF), a c-Neu ligand, or to culturing on an extracellular matrix (ECM) comprised solely of fibronectin, the stem cells preferentially generate glial cells. Stem cells have also been isolated from the adult sriatum. Striatal stem cells divide in response to a non-adhesive ECM and epidermal growth factor (EGF) to generate both astrocytes and neurons (Reynolds and Weiss, 1992).

Although homologues of *Drosophila* proneural and neurogenic loci have been isolated, it is not always clear that the genes subserve the same functions in vertebrates.

Notch counterparts exist in *Xenopus* (*Xotch*) (Coffman et. al., 1990), rat (Weinmaster et. al., 1991; Weinmaster et. al., 1992), and mouse (Reaume et. al., 1992). In each species, expression of the Notch family member is enriched in the developing nervous system. Several experiments have suggested that vertebrate Notch, unlike its *Drosophila* counterpart, may not act simply to repress neuronal fate. Animals homozygous for null mutations in *Notch1* die as embryos, and a substantial amount of cell death is seen in the central and peripheral nervous systems (Swiatek et. al., 1994). Prior to the observed degeneration, the nervous systems of *Notch1* mutants do not appear

hypertrophic. *Notch* expression has been carefully analyzed in the developing cerebral cortex (Chenn and McConnell, 1995). Cortical NBs divide asymmetrically to generate immature neurons and other NBs. *Notch* is asymmetrically expressed in these dividing precursors, and is selectively inherited by the neuron. In this respect, *Notch* resembles the asymmetrically distributed numb protein in *Drosophila* sensory precursors (see above). It is not expressed uniformly in the cortical progenitors.

The effects of dominant, gain-of-function forms of *Notch* were examined in both *Xenopus* and in mammalian cell lines. Activated *Xotch* appears to delay differentiation of a variety of tissues in *Xenopus* embryos, many of which go on to develop normally after the levels of the mutant protein fall off (Coffman et. al., 1993). The effects of this mutant form of *Notch* were analyzed in a mouse cell line, P19, which can normally be induced to generate myocytes, neurons, or glial cells (Nye et. al., 1994). In the presence of activated *Notch*, P19 cells form neither myocytes nor neurons, but can still be induced to generate glial like cells. Activated *Notch* also represses myogenesis *in vivo* within *Xenopus* embryos (Kopan et. al., 1994). When activated *Notch* was examined in HeLa cells, it was found to activate transcription by interacting directly with a human homologue of *Suppressor of Hairless* (Jarriault et. al., 1995). Although activated forms of *Notch* can suppress neuronal fates, it is not clear that this is its normal function. The data is consistent with *Notch* acting, instead, in the early steps of neuronal development (but see below). A better understanding of *Notch* function will require the construction of a double mutant, in which both the *Notch1* and *Notch2* genes are missing.

Recent experiments with *Delta* homologues are easily reconciled with the *Drosophila* literature. *Xenopus* (*X-Delta-1*) and chick (*C-Delta-1*) homologues are expressed in presumptive neurons (Chitnis et. al., 1995; Henrique et. al., 1995). Overexpression of X-Delta-1 results in an anti-neurogenic phenotype (Chitnis et. al., 1995). In this study, an activated form of *Notch* also resulted in an anti-neurogenic phenotype. When a mutant form of *Delta* which lacks its intracellular domain is expressed, a neurogenic phenotype is observed. These data indicate that the vertebrate *Notch* and *Delta* signaling pathways can act to restrict neuronal fate.

One vertebrate homologue of the *Drosophila achaete-scute* (AS-C) genes, MASH-1 (Johnson et. al., 1990), is expressed in subsets of cells within the CNS

and PNS (Lo et. al., 1991; Guillemont et. al, 1993). Animals mutant for MASH-1 die shortly after birth. The CNS appears normal in these mutants, but fewer olfactory neuron progenitors are found, and the differentiation of neural crest into sympathetic ganglia is blocked (Guillemont et. al., 1993). The *Xenopus* homologue, XASH-1, is expressed more widely in the CNS, and is not seen in neural crest derivatives (Ferreiro et. al., 1992). How well the function of MASH-1 parallels AS-C function is unclear. MASH-1 appears to act as a "proneural" gene for at least some of the olfactory neuronal precursors. In the neural crest, however, the gene appears to affect the early stages of neuronal differentiation, acting downstream of the decision to become a neural precursor.

Other bHLH proteins are likely to be involved in the specification of neuronal fate. Two murine homologues of the *atonal* locus have been identified. MATH-1 is 70% identical to *ato* through the bHLH domain, and is expressed in the dorsal part of the CNS (Akazawa et. al., 1995). MATH-2 is 53% similar to *ato* within the bHLH domain, and is expressed more widely than MATH-1 (Shimizu et. al., 1995). An additional bHLH protein, NeuroD, has been identified in *Xenopus* (Lee et. al., 1995). NeuroD is expressed in subsets of neural precursors within the CNS and PNS, and its ectopic expression is sufficient to transform epidermal cells into neurons.

Neural-glial interactions

The earliest references to glia were made in the late 1800s, when a German pathologist, Virchow, described "nervenkitt", or, a "binding substance" in which the "nerve elements are embedded" (reviewed in Young, 1991). The word "kitt" was translated as "glue", and the word "glia" is derived from the Greek. The first morphological descriptions of glia came from Golgi (1870) and Cajal (1909 and 1913). Mature glia may exhibit elaborate extensions referred to as "branches", or "processes". Glia are often classified based on their morphology. A common distinction is drawn between vertebrate glia with a "stellate" morphology (astrocytes) and glia with few processes (oligodendrocytes). Recently, a classification of *Drosophila* glia has been published (Ito et. al., 1995). Communication between glia and neurons is essential to both the development and the function of nervous systems.

Effects on proliferation, differentiation and survival

One of the best described examples of glial-neural signaling is found in the vertebrate PNS, between peripheral sensory axons and their associated glial cells which are called Schwann cells (reviewed in Bray et. al., 1981; Jessen and Mirsky, 1992). One class of Schwann cells ensheaths single axons in multiple, concentric layers of myelin, a substance composed of alternating layers of a compressed lipid bilayer and protein (Fig. 4A). Myelination increases the rate at which an action potential is conducted. A second class of Schwann cells does not generate myelin, but extends processes into nerve fibers to separate axons into smaller bundles (Fig. 4B). Although mature Schwann cells are morphologically and molecularly distinct from each other, they are generated by a common, neural crest derived progenitor (reviewed in Jessen and Mirsky, 1992 and see below). Contact between these glial precursors and peripheral axons occurs in several, genetically distinct steps (reviewed in Bray et. al., 1981; Jessen and Mirsky, 1992). Some mutations disrupt the initial interactions between Schwann cells and axons, while other mutations disrupt myelination.

Recently, the Schwann cell progenitor has been described (Jessen et. al., 1994). Schwann cell precursors are molecularly distinct from either type of mature Scwhann cell. The precursors are also unique in that they require an axonal cue for survival *in vitro*. In the presence of media conditioned by peripheral nerves, Schwann cell precursors will mature *in vitro*. The signal from the axons appears to be neu differentiation factor (NDF) (Dong et. al., 1995). A single, alternatively spliced gene gives rise to NDF and a number of related factors, notably, glial growth factor (GGF) (Wen et. al., 1994). NDF promotes the survival and differentiation of Schwann cell precursors *in vitro*. *In vivo*, immature Schwann cells express an NDF receptor, while the associated sensory axons express NDF itself (Dong et. al., 1995).

Communication between glia and neurons also occurs within the vertebrate CNS. Retinal ganglion cell survival is mediated, initially, by the adjacent Müller glial cells (Armson et. al., 1987). Over time, the ganglion cells lose their dependence on the Müller glia and begin to rely on a target derived trophic factor. As retinal ganglion cell axons grow into the brain, they fasiculate to form the optic nerve. Several classes of glial cells are associated

with the optic nerve. Electrical activity in the retinal axons induces one class of optic nerve glial cell, the type I astrocyte, to release a mitogen (Barres and Raff, 1993; reviewed in Barres et. al., 1994). The mitogen triggers the proliferation of another glial cell type, the oligodendrocyte. Once oligodendrocytes are born, an additional axonal signal is required to promote their survival (reviewed in Barres and Raff, 1994). Within the brain, a membrane bound astrocyte signal inhibits the proliferation of cerebellar granule neuron precursors (Gao et. al., 1991). In both the cerebellum and the hippocampus, neurons induce the differentiation of immature glial cells (Gasser and Hatten, 1990; reviewed in Hatten, 1990). A novel, glial derived growth factor with some homology to the TGFβ superfamily supports the survival of CNS neurons *in vitro* (Lin et. al., 1993).

At least two neural-glial signals have been described in the embryonic CNS of *Drosophila*. The *pointed* (*pnt*) locus acts cell autonomously to direct the differentiation of the midline glia (Klämbt, 1993). Ectopic expression of one of the *pnt* transcripts is sufficient to induce supernumerary glial cells, which, in turn, appear to induce the ectopic expression of neuronal antigens in adjacent cells (Klaes, et. al., 1994). Approximately 50% of the midline glia are eliminated towards the end of embryogenesis by apoptosis (Sonnenfeld and Jacobs, 1995). Survival appears to require contact with axon fibers, in a manner reminiscent of oligodendrocytes within the rat optic nerve (see above).

In the post-embryonic CNS of *Drosophila*, a glial-derived secreted protein, *anachronism* (*ana*), regulates the quiescent state of larval neuroblasts (NBs) (Ebens et. al., 1993). In the absence of *ana*, NBs begin to proliferate earlier than they would in wild type animals. In adult animals, glia provide a survival factor required by neighboring neurons (Xiong and Montell, 1995). When glial differentiation is flawed in the visual system, both glial and neuronal components degenerate.

In the *Drosophila* PNS, wing sensory neurons appear to influence the development of a class of lineally distinct glial cells. The sensory neurons and the glia which ensheath the sensory nerve appear to derive from different progenitors (Giangrande et. al., 1993; Giangrande, 1994). Proneural gene expression is required in the sensory neuron, or its precursor, for the development of the nerve-associated glia, which implies a neuron to glia

signal (Giangrande, 1995).

Migration

Cells in the developing nervous system may migrate from their point of origin to their final positions. Neurons and glia will use each other as migration substrata, and may provide signals required for the termination of migration.

In the vertebrate cerebellum, granule neurons are born in a proliferation zone called the external germinal layer (EGL), and migrate to their final positions within the internal granule cell layer (IGL) along a specific class of glial cells, the Bergmann glia (see Fig. 5A) (reviewed in Hatten, 1990; Hatten and Heinz, 1995). Granule neurons express a cell surface glycoprotein, astrotactin (Edmondson et. al., 1988), which appears to mediate their migration along the Bergmann glia (Fishell and Hatten, 1991). In the hippocampus, neuronal migration along glial cells resembles the interaction between cerebellar neurons and Bergmann glia (Gasser and Hatten, 1990).

During the development of the vertebrate peripheral nervous system, a subset of neural crest cells colonize the ventral root and differentiate into glial cells (Fig. 5B). One study has reported that motor axons from the spinal cord are required to correctly position this population of crest cells (Bhattacharyya et. al., 1994). In the absence of these axons, crest cells appear to reach the ventral root and continue to migrate.

In *Drosophila*, glia in the wing disc migrate along a sensory nerve (Giangrande, 1994). Glia also migrate into the developing eye disc from the optic stalk, and appear to migrate along bundles of photoreceptor axons (Choi and Benzer, 1994). At the embryonic midline, two sets of axon fascicles, called commissures, separate the longitudinal connectives (Fig. 5C) (Klämbt et. al., 1991). The commissures are initially adjacent to one another, and are separated by the posterior migration of one pair of midline glia.

Boundaries

Glial cells frequently form "barriers" which surround the boundaries of a developing neuropil (reviewed in Steindler, 1993). In vertebrates, the best described example of a glial boundary is seen in the developing somatosensory cortex. In some animals, the primary sensory organs of the

face are whiskers (reviewed in Woolsey, 1990). Sensory information is relayed to the trigeminal ganglia in the brainstem, and then to the thalamus, and then, finally, to the cortex (Fig. 6A). The pattern of the whiskers is maintained in each region of the brain. Afferent inputs and their target neurons are segregated into "barreletts" in the brainstem, "barreloids" in the thalamus, and "barrels" in the cortex. The cortical barrel field forms in response to afferent input (reviewed in Faissner and Steindler, 1995). Glia within each barrel express cytotactin, while the target neurons express a ligand for cytotactin (Crossin et. al., 1989). Expression of these and other molecules facilitates local neural-glial adhesion, and the creation of barrel "walls". It is believed these walls confine neurite outgrowth and synapse formation to the area within the barrels, thereby maintaining the topography of the whisker map.

Descriptive studies have indicated that glial cells are found along the vertebrate CNS midline (reviewed in Steindler, 1993). Glia are found between the cerebral hemispheres (Silver et. al., 1993), and are tightly associated with each other at the roof plate of the spinal cord (Snow et. al., 1990). It has been suggested that these glia function to prevent axons from crossing the midline at inappropriate times or locations (reviewed in Steindler, 1993 and Silver, 1993).

In the moth, sensory axons from the antennae terminate in the antennal lobe within discrete regions termed "glomeruli" (reviewed in Tolbert and Oland, 1989; Tolbert and Oland, 1990). Mature glomeruli consist of sensory axon termini, axons from antennal lobe neurons, and a ring of glial cells (Fig. 6B). Prior to innervation, glia uniformly surround the antennal lobe neuropil. Ingrowing axons appear to recruit the glia into distinct units, in which they surround clusters of sensory axons. Following glial migration into the proto-glomeruli, the second order antennal lobe neurons extend axons into the glomeruli, and synapse with their sensory neuron partners. It has been suggested that the glomeruli are analogous to cortical barrels, and function to ensure that afferent inputs contact their appropriate targets. Glomeruli are also seen within the vertebrate olfactory bulb (Valverde and Lopez-Mascaraque, 1991 and Gonzalez et. al., 1993). Glia associated with these glomeruli synthesize ECM material that appears to serve as the glomeruli "walls".

In the grasshopper, glial cells segregate proliferating brain neuroblasts into clusters (Boyan et. al., 1995). Axons grow in between these clusters, or along the glial cells themselves.

Axon guidance

Aside from their roles in cell migration and boundary formation, glial cells may provide positional cues to axons during their pathfinding phase.

The vertebrate cerebral hemispheres are connected by defined fiber tracts, one of which is the corpus callosum. Prior to the formation of the corpus callosum, a glial "sling" forms (Silver et. al., 1982). The axons which pioneer the corpus callosum use the glial sling as a bridge across the midline. In mutants which lack these glial cells, or in animals in which the "sling" is lesioned, the callosal axons do not cross the cerebral midline. Callosal axons are also associated with glial cells after they've crossed the midline, and could potentially use these glia as a pathways (Norris and Kalil, 1991).

In vitro analyses have indicated that vertebrate glia are capable of providing both attractive and inhibitory cues to axons. An example of an inhibitory cue is seen in the optic tectum. In the *Xenopus* visual system, temporal retinal axons grow into the anterior tectum and nasal retinal axons grow into the posterior tectum (Fig. 7A). When retinal neurons are co-cultured with tectal glial cells, temporal neurites collapse upon contact with posterior glial cells (Jonhston and Gooday, 1991; see below). In contrast, astrocytes isolated from the cerebral cortex support neurite outgrowth from cerebellar and spinal cord neurons (Noble et. al., 1984).

In *Drosophila*, glial cells prefigure axon pathways within the ventral nerve cord (Jacobs and Goodman, 1989a). Midline glia are associated with the commissures, longitudinal glia are associated with the longitudinal connectives, and the segment boundary glial cell (SBC) is associated with the intersegmental nerve (ISN) (Fig. 7B). Each class of glial cell appears to provide the template for subsequent axon tract formation. The ISN is formed by a subset of axons which extends laterally from the longitudinal connectives (Bastiani and Goodman, 1986; Jacobs and Goodman, 1989). The ISN pioneers turn laterally upon contacting the SBC. If the SBC is ablated, the ISN pioneers fail to turn, remaining associated with the longitudinal connectives (Bastiani and Goodman, 1986). Glial cells are seen along the trajectory of the

longitudinal connectives, and mutations which disrupt the development of these glia show subsequent disruptions of the longitudinal tracts (Jacobs, 1993). Commissural axons cross the midline at stereotypic positions within each segment, contacting both midline neurons and glia (Klämbt et. al., 1991). In the absence of the midline cells, the commissures do not form (Menne and Klämbt, 1994). Glial cells are also associated with nerves in more dorsal aspects of the ventral nerve cord. The transverse nerves (TNs) extend from the CNS into the periphery (Gorczyca et. al., 1994). A TN grows as a single fascicle until it reaches a particular glial cell (exit glial cells), at which point it bifurcates. In a mutant, *tinman* (*tin*), which lacks the TN exit glial cells, the TN nerves fails to make the correct extensions into the periphery.

Other functions

Glial cell tight junctions form the insect "blood-brain" barrier (reviewed in Lane, 1981). When the blood-brain barrier is disrupted, as in the *gliotactin* mutant, neuronal activity is impaired by the ionic concentration of the hemolymph (Auld et. al., 1995). Glia also appear to be able to function in setting circadian rhythms. The *Drosophila period (per)* gene encodes a clock protein, and expression of *per* in glia within the adult head is sufficient for some rhythmic behavior (Ewer et. al., 1992). Finally, glial cells enhance an animal's "quality of life". In the absence of *drop dead* function, glia in the *Drosophila* head develop poorly. Although their behavior is normal shortly after eclosion, *drop-dead* mutants develop locomotor difficulties and die within 1-2 weeks (Buchanan and Benzer, 1993). An extensive literature exists regarding the role of glia in regeneration (reviewed in Bähr and Bonhoeffer, 1994 and Sivron and Schwartz, 1995) and in extracellular buffering (see, for e.g.., Reichenbach, 1991; Schlue et. al., 1991).

Axon pathfinding

In addition to relying on glial cells for guidance cues, axons navigate by using information provided by neurons and other cell types along their trajectory. Guidance cues may be inhibitory or growth promoting, and may be found on the surface of a cell, deposited in the extracellular matrix, or provided by diffusible cues (reviewed in Goodman and Schatz, 1993, Baier

and Bohnhoeffer, 1994; Kennedy and Tessier-Lavigne, 1995; Dodd and Schuchardt, 1995; Keynes and Cook, 1995; and Garrity and Zipursky, 1995). This discussion will be restricted to the early stages of axon guidance, and will refer to neither the molecular details of growth cone response to extracellular cues (reviewed in Lander, 1990; and see Kuhn et. al., 1995, Fan and Raper, 1995), nor to studies regarding the refinement of projection patterns via electrical activity (reviewed in Goodman and Schatz, 1993).

Optic chiasm

Prior to entering the brain, retinal ganglion axons extend through the optic chiasm (Fig. 7C). In rodents, most retinal ganglion cells project to the contralateral side of the brain, while the projections of ganglion cells located in the ventral-temporal region are ipsilateral (reviewed in Stretavan, 1993). This pattern could be the result of retinal axons making distinct pathway choices, or it could reflect the selective elimination of axons which have projected in an alternative manner. To distinguish between these possibilities, two groups analyzed retinal ganglion projections in mouse embryos and found two distinct projection patterns (Stretavan, 1990; Godement et. al., 1990). As in the adult, most ganglion cells project contralaterally, while ganglion cells located in the ventral temporal region of the developing retina project ipsilaterally. Analyses of growth cone behavior at the chiasm indicate that ipsilateral projections approach the midline, make a "sharp" turn, and then project ipsilaterally (Godement et. al., 1990; Stretavan and Reichardt, 1993). These data indicate that retinal axons choose specific pathways. The cues which underlie the decision to turn are centered at the midline, and appear to be membrane associated (Wizenmann et. al., 1993). Retinal ganglion cells appear to avoid a V-shaped cluster of neurons located at the chiasm (Stretavan et. al., 1994). Contralateral projections extend through the opening of the V, while ipsilateral projections turn back towards the ipsilateral optic tract. The inhibitory affect of the V cluster may be attributed, in part, to its expression of a cell surface molecule, CD44 (Stretavan et. al., 1994).

Optic tectum

Experiments by Sperry indicated that retinal axons correctly identify their

targets in the optic tectum regardless of whether or not ganglion cell orientation in the eye has been altered (Sperry, 1963). This indicated that axons do not simply project to the first available position within a target field. Based on this data, the "chemoaffinity" hypothesis was proposed. In this model, an axon finds its target because both cells are distinctly labeled. Position within a target field may be specified by expression of unique molecules at each point in the field, or by a gradient of expression of a few molecules. Recent work has demonstrated the existence of gradients within the developing retina and tectum in lower vertebrates.

In the retina, axons from nasal ganglion cells project into the posterior tectum, while temporal axons project into the anterior tectum (Fig. 7A) (reviewed in Tessier-Lavigne, 1995). *In vitro* analyses indicated that temporal axons are repelled by posterior tectal membranes, while nasal axons are not (Walter et. al., 1987; Cox et. al., 1990; Wizenmann et. al., 1993). The selective inhibitory activity can function as a gradient *in vitro* (Baier and Bonhoeffer, 1992). Recently, a gene encoding an inhibitory activity has been cloned from the tectum. The inhibitory activity, referred to as RAGS (repulsive axon guidance signal) (Drescher et. al., 1995), is highly homologous to the ligands for the Eph receptors, a class of receptor tyrosine kinases (Cheng and Flanagan, 1994). RAGS is expressed in a gradient within tectal glial cells, with the highest concentrations located in the posterior part of the tissue. Given that RAGS does not differentially affect temporal and nasal axons *in vitro*, there may be additional molecules required to confer positional specificity. A related Eph ligand, ELF-1, is expressed in a pattern similar to the RAGS pattern (Cheng et. al., 1995). Interestingly, an Eph receptor, Mek4, is expressed in a similar gradient within the developing retina. The highest levels of Mek4 are seen in temporal retinal ganglion cells. This class of receptors and ligands appears to provide part of the specificity seen in retinal-tectal projections.

Cortex

In higher vertebrates, retinal axons project into the lateral geniculate nucleus (LGN) and the superior colliculus. The LGN is the site at which visual information begins to be processed. Projections from the LGN into the visual cortex are called thalamic projections. These axons innervate the

cerebral cortex prior to the positioning of their target neurons (Fig. 7D) (reviewed in Allendoerfer and Schatz, 1994). Thalamic axons accumulate beneath the visual cortex in a region called the subplate, and rely on transient targets, called the subplate neurons, to correctly position them beneath the cortical layers (McConnell et. al., 1989; Ghosh et. al., 1990). When the subplate neurons are ablated, thalamic axons bypass their targets. The growth of thalamic axons from the subplate into their appropriate cortical layer appears to be influenced by membrane bound molecules (Gotz et. al., 1992).

Spinal cord

Axonal trajectories are not simply established by the polarity of axon outgrowth from neuronal cell bodies. Within the vertebrate spinal cord, axons respond differentially to environmental cues. Spinal cord axons project along either a longitudinal or a circumferential trajectory (Fig. 8) (reviewed in Colamarino and Tessier-Lavigne, 1995). Circumferential axons either cross the spinal cord at the floor plate (commissural axons) or turn at right angles to fasiculate with a longitudinal tract (association axons).

In vitro analyses with rat explants indicated that the floor plate contains a chemoattractant for commissural axons (Tessier-Lavigne et. al., 1988; Placzek et. al., 1990). A chemoattractant activity with similar properties was identified in embryonic chick extracts (Serafini et. al., 1994). Two diffusible proteins, netrin-1 and netrin-2, were isolated from chick extracts. The expression of netrin-1 is restricted to the floor plate, while netrin-2 is seen in the ventral 2/3 of the spinal cord (Kennedy et. al., 1994). Expression of either netrin in heterologous cells is sufficient to re-orient the growth of spinal cord commissural axons *in vitro*. The netrins are 50% identical to a *C. elegans* gene, UNC-6, which is required for the correct guidance of axons and migrating cells (Ishii et. al., 1992).

Once commissural axons reach the floor plate, they appear to rely on local cues to guide them across the floorplate (reviewed in Colamarino and Tessier-Lavigne, 1995). In the chick embryo, two cell surface adhesion molecules have been implicated in midline crossing (Stoeckli and Landmesser, 1995). Axonin-1 is expressed by the commissural neurons and Nr-CAM is expressed by the floor plate. The addition of blocking antibodies to either molecule leads to routing errors, resulting in the failure of many

commissural axons to cross the midline.

In contrast to its interaction with commissural axons, the floor plate is inhibitory to motor axons (Guthrie and Pini, 1995; Colamarino and Tessier-Lavigne, 1995). Motor neuron cell bodies are found within the ventral spinal cord, and their axons exit the spinal cord at multiple points to project into the periphery (Fig. 9). Several classes of motor axons will alter their exit trajectories, *in vivo*, to avoid an ectopic floor plate (Guthrie and Pini, 1995). When co-cultured *in vitro*, motor axons will avoid floor plate explants. Heterologous cells expressing netrin-1 are sufficient to repel motor axons *in vitro* (Colamarino and Tessier-Lavigne, 1995). An analysis of the netrin receptors will facilitate an understanding of the differential response shown by motor and commissural axons.

One class of axons which innervate the spinal cord from the periphery are sensory axons from the dorsal root ganglion (DRG) (Fig. 8). Phenotypically distinct classes of sensory neurons are found within the DRG (see Hamburger and Levi-Montalcini, 1949; Ernfors et. al., 1994), and each class projects to a discrete region of the spinal cord. In particular, neurons that respond to NGF terminate in the dorsal region of the spinal cord, while NT-3 responsive neurons terminate in the ventral spinal cord. These two types of neurons show different responses to ventral spinal cord explants (Messersmith et. al., 1995). Ventral spinal cord inhibits the outgrowth of axons from NGF responsive neurons, but does not affect NT-3 neurons. This inhibitory signal is likely to correspond to Semaphorin III, which shows restricted expression in the ventral spinal cord. Sema III is a member of the collapsin/semaphorin family (reviewed in Dodd and Schuchardt, 1995). These genes encode secreted or transmembrane bound molecules with unique "semaphorin" domains, Ig-like domains, and basic C termini (Kolodkin et. al., 1993; Luo et. al., 1993; Luo et. al., 1995; Puschel et. al., 1995).

Vertebrate members of the family will subsequently be referred to as "collapsins", while invertebrate members will be referred to as "semaphorins" (see below).

The first collapsin was identified biochemically, based on its ability to cause DRG axons to "collapse" on contact in an *in vitro* assay (Luo et. al., 1993). Additional members have been cloned by homology (Luo et. al., 1995; Puschel et. al., 1995). The collapsins are expressed within the developing

nervous system, and several have been demonstrated to have a "collapsing" activity *in vitro*.

Insect imaginal discs

Peripheral axons grow into the insect CNS along established axon tracts which are called pioneer neurons (Bate, 1976; Bentley and Keshishian, 1982; Jan et. al., 1985). Pioneer neurons themselves require guidepost cells to form the more distal parts of their trajectory (Bently and Caudy, 1983). Upon the ablation of these guidepost neurons, pioneer axons fail to complete their projections. The polarity and initial extension of a pioneer axon appears to rely solely on the epithelium (Blair and Palka, 1985; Lefcort and Bentley, 1987; Blair et. al., 1987). In some parts of the *Drosophila* nervous system, pioneer axons are not absolutely required for pathfinding, but appear to increase the speed and fidelity with which axons extend along their trajectories (Lin et. al., 1995).

Insect central nervous system

Patterns of axonal projections in the grasshopper CNS have been well described (Raper et. al., 1983a; Raper et. al., 1983b; Bastiani et. al., 1986; du Lac et. al., 1986). These studies demonstrated that axons follow stereotyped pathways, and that growth cones can respond quite differently to the same environmental cues (Raper et. al., 1983 a,b). If an axon's normal pathway is ablated, it does not choose another one (Bastiani et. al., 1986; du Lac et. al., 1986). Axons appear to behave quite similarly in *Drosophila* embryos (Jacobs and Goodman, 1989b).

In order to identify the molecules which confer specificity to axon pathfinding, monoclonal antibodies were screened to identify antibodies which label subsets of the grasshopper CNS. Four genes, and their *Drosophila* homologues, were identified (reviewed in Grenningloh and Goodman, 1992). Fasiclin (fas) I-III and Neuroglian encode cell surface adhesion molecules. Fas I is homophilic (Elkins et. a., 1990a), and mutations, in combination with mutations in the Ableson tyrosine kinase receptor, cause defects in the commissural tracts (Elkins et. al., 1990b). Fas II function is required in three different axon pathways. In loss of function mutations, fas

II growth cones stall along their pathway (Grenningloh et. al., 1991) and axon tracts appear defasiculated (Lin et. al., 1994). Conversely, overexpression of fas II leads to the fusion of axon tracts. In the absence of fas III function, the fas III$^+$ motor neurons correctly innervate their targets, suggesting some functional redundancy (Chiba et. al., 1995). However, ectopic expression of fas III disrupts correct pathfinding, indicating that a restricted expression pattern is important for its function.

Semaphorin I was originally called Fasiclin IV, and is a transmembrane protein (Kolodkin et. al., 1992). An *in vitro* analysis with blocking antibodies to Semaphorin I (Sema-I) indicated that the molecule acts to prevent defasiculation and inappropriate branching. In the process of identifying two *Drosophila* homologues, D-Sema-I and D-Sema-II, it became apparent that all three genes were members of the collapsin gene family (see above)(Kolodkin et. al., 1993). Animals which are null for D-Sema-II have no obvious structural defects in the nervous system, however the animals display severe motor defects and die shortly after eclosion. When D-Sema-II is overexpressed, subsets of motorneurons fail to choose targets, appropriate or otherwise (Matthes et. al., 1995). This data indicates that D-Sema-II acts as an inhibitory molecule, in much the same way that the vertebrate collapsins do.

Genetic screens have been carried out in *Drosophila* to identify genes required for axonal projections across the midline and genes which act during motorneuron pathfinding. The midline screen led to the isolation of two mutations with opposite affects on commissural pathfinding (Seeger et. al., 1993). In *commissureless* (*comm*) mutants, most commissural axons fail to cross the midline. In *roundabout* (*robo*) mutants, many axons inappropriately cross the midline. The motorneuron screen identified several mutations which disrupt axonal projections at distinct points along their trajectories (Van Vactor et. al., 1993). These data indicate that motorneuron axons encounter several choice points between the CNS and their targets.

Development of the *Drosophila* visual system

The adult *Drosophila* visual system consists of the compound eyes and bilateral optic ganglia (reviewed in Meinertzhagen and Hanson, 1993) (Fig.

9A). The progenitors of the retina and the optic ganglia, which arise from the dorsal ectoderm, are segregated from each other during embryogenesis (Green et. al., 1993; Younossi-Hartenstein et. al., 1993). Early development of both the eye and the optic ganglia requires a homeodomain protein, *sine oculis*. (Cheyette et. al., 1994; Serikaku and O'Tousa, 1994). An additional locus, *eyeless*, encodes a transcription factor that is sufficient to induce ectopic eye development (Quiring et. al., 1994; Halder et. al., 1995). The compound eye is comprised of 800 unit eyes, or, "ommatidium", each of which contains eight photoreceptor neurons (R-cells) and 6 support cells (Fig. 9B). The outer R-cells (R1-6) project into the first optic ganglion, or, lamina. The inner photoreceptors (R7,8) project into the medulla.

Summary of retinal development

Retinal differentiation begins in late third instar life, as a morphogenetic furrow moves across the eye disc from its posterior to its anterior borders (Ready, Hanson and Benzer, 1976). The eye develops in a re-iterative manner, coincident with furrow progression, such that the oldest ommatidia are at the posterior border of the disc. The disc is unpatterned anterior to the furrow, with the exception of a single mitotic zone. Divisions within the anterior mitotic zone give rise to the first cells to be incorporated into the nascent ommatidia following the passage of the furrow (Wolff and Ready, 1991). After ommatidial preclusters emerge from the furrow, a second mitotic zone generates the remaining cells of each ommatidium (Fig. 10). Cells within an ommatidium are not clonally related (Ready et. al., 1976; Lawrence and Green, 1979). The differentiation of cells within an ommatidium occurs in a stereotyped fashion. R8 is the first cell to develop within the ommatidium, and it is followed by R2 and R5, then R3 and R4, R1 and R6, and, finally, R7 (Tomlinson and Ready, 1987). Following the specification of the R8 cell (see below), R-cell development is controlled by a series of local cell-cell interactions (reviewed in Zipursky and Rubin, 1994; and see Kimmel et. al., 1990; Heberlein and Rubin, 1991; Dickson et. al., 1995).

The progression of the furrow, unlike its initiation, requires signaling from the developing photoreceptors themselves (Fig 10; reviewed in Heberlein and Moses, 1995). Photoreceptors express a secreted protein,

hedgehog (*hh*). The *hh* signal induces the expression of *decapentaplegic* (*dpp*), a TGFβ1 family member, within the morphogenetic furrow (Heberlein et. al., 1993; Ma et. al., 1993). *dpp* expression is required for furrow movement, and is restricted to the furrow by two bHLH proteins. Both *hairy* (*h*) and *extramachrochaete* (*emc*) are expressed anterior to the furrow, and repress *dpp* expression (Brown et. al., 1995).

Neuronal identity is conferred on the photoreceptors by the expression of the proneural gene, *atonal* (*ato*) in the ommatidial founder cell, R8 (Jarman et. al., 1994). *ato* expression begins anterior to the furrow and is uniform along the dorsal/ventral axis (Fig. 10). Following the passage of the furrow, *ato* expression is restricted to the presumptive R8 cell. The restriction of *ato* expression requires three neurogenic loci: *Notch*, *Delta* and *scabrous* (Baker and Zitron, 1995). Clusters of cells express *scabrous* anterior to the furrow. Subsequently, *N* and *Dl* restrict *sca* and *ato* expression to single cells, which develop into the founder cells of each ommatidium.

Photoreceptor cells mature shortly after their generation (Tomlinson and Ready, 1987), and photoreceptor axons (R-axons) project to the basal portion of the eye disc and through an epithelial sheath, called the optic stalk, into the developing brain (reviewed in Kunes and Steller, 1993 and Meinertzhagen and Hanson, 1993). R-axons are capable of projecting to the correct region of their target field in the absence of cues from other photoreceptors, suggesting that they may rely on positional information within the brain (Kunes et. al., 1993; Ashley and Katz, 1994). This idea is supported by recent experiments which demonstrated that the retinal target field is organized, in part, by two secreted proteins, *wingless* (*wg*) and *dpp* (Kaphingst and Kunes, 1994). In the absence of either *wg* or *dpp* function in the brain, retinal axons do not correctly innervate the target area. Retinal projections may be disrupted at several discrete points. Mutations have been identified which block R-axons from exiting the eye disc, projecting retinotopically, and terminating within the correct ganglion (Martin et. al., 1995). This data suggests that, like motor axons in the *Drosophila* CNS (see above), the R-axons rely on a series of cues along their trajectory. One gene has been identified which acts within the target field to affect retinal projections. The *giant lens* (*gil*) locus encodes a secreted protein, and in the absence of its function, retinal axons do not

penetrate the brain's surface (Kretzschmar et. al., 1992; Brunner et. al., 1994). The cellular origins and the molecular identities of R-axon guidance cues remain largely unknown.

Summary of optic lobe development

The optic lobes are generated by 30-40 neuroblasts, which are located in the posterior brain hemispheres and resume proliferation early in first instar (Hofbauer and Campos-Ortega, 1990). Two proliferation zones are established on the brain's surface, and each zone "sheds" daughter cells medially (White and Kankel, 1978). The larger zone is called the outer proliferation center (OPC), and generates cells in the lamina and medulla. The smaller zone is called the inner proliferation center (IPC), and generates cells of the lobula complex. A third proliferation zone appears in late third instar larvae (see below).

The first cells to differentiate within the optic lobes are of embryonic origin. These cells, referred to as the optic lobe pioneers (OLPs), are found at the base of the eye stalk and project into the central brain (Tix et. al., 1989). Cells of larval origin differentiate in the order in which they are born (White and Kankel, 1978). The differentiation of the lobula and the medulla occurs before lamina development, and does not require retinal input (Fischbach, 1983).

The re-activation of optic lobe NB proliferation appears to be regulated, in part, by the products of the *anachronism* (*ana*) and *terribly reduced optic lobes* (*trol*) loci (Datta, 1995). In *ana* mutants, NB divisions begin precociously (Ebens et. al., 1993), while, in *trol* larvae, NB proliferation is severely impaired (Datta and Kankel, 1992). *ana* appears to act downstream of *trol*. The proliferation of optic lobe NBs is also controlled by the *l (1) optic ganglion reduced* (*l(1)ogre*) and *minibrain* loci (Lipshitz and Kankel, 1985; Tejedor et. al., 1995). The *l(1) decreased volume optic lobes* (*l(1)devl*) locus is required for the correct organization of the optic lobe, but does not appear to affect cell proliferation (Datta and Kankel, 1992). Similarly, mutations in the *optomotor-blind* (*opt*) (Pflugfelder et. al., 1992) and *asense* (Gonzalez et. al., 1989) loci appear to disrupt optic lobe neuropil without noticeably affecting cell number. One gene has been cloned which is required for axon pathfinding between optic ganglia. *irregular chiasm C* (*irreC*) encodes a

homophilic cell adhesion molecule which is expressed in the neuropils of all three optic ganglia (Ramos et. al., 1993; Schneider et. al., 1995). Either mutations in *irre-C*, or overexpression of the protein, lead to aberrant axonal projections. The *Blackpatch* (*Bpt*) locus affects cell survival in the visual system (Duus et. al., 1992). At a low penetrance, *Bpt* mutants show degeneration of the retina, lamina and medulla. Mutations in the *small-optic-lobes* (*sol*) gene acts autonomously in the medulla, and affect both the medulla and the lobula. In *sol* mutants, cells of the medulla and lobula degenerate shortly after they are generated (Fischbach and Technau, 1984). Few genes which affect lamina development by acting autonomously within the ganglion have been identified (but see below).

R-axons contact the optic ganglia midway through the third instar (reviewed in Meinertzhagen, 1973). At this time, a third proliferation zone is seen at the base of the optic stalk (White and Kankel, 1978). Retinal input induces these divisions, which generate the lamina neurons (Selleck and Steller, 1991; Selleck et. al., 1992). Lamina neurogenesis requires the *division abnormally delayed* (*dally*) locus (Nakato et. al., 1995). *dally* encodes a putative proteoglycan, which is expressed by the neuronal progenitors.

It was clear from an earlier mosaic study that the R-axons also communicate, in some fashion, with the lamina glial cells. In adult animals in which the retina is smaller or missing altogether, all of the underlying lamina is correspondingly reduced in size (Fischbach and Technau, 1984). Several mechanisms would explain the absence of the lamina glia in eyeless flies. One possibility is that the R-axons trigger lamina gliogenesis as well as lamina neurogenesis. A second possibility is that retinal input is required for the maturation of the lamina glia. Finally, it is possible that retinal innervation provides some sort of trophic factor that is required for lamina glial survival.

The aim of this thesis is twofold: (1) to describe the cellular interactions between retinal axons and glial cells within the first optic ganglion of *Drosophila*, and (2) to molecularly and genetically describe a novel gene, *lamina ancestor* (*lama*), which is expressed in the first optic ganglion's progenitors.

References

Anderson, D. J. (1989). The neural crest cell lineage problem: neuropoiesis? *Neuron.* **3:** 1-12.

Auld, V. J., Fetter, R. D., Broadie, K. and Goodman, C. S. (1995). Gliotactin, a novel transmembrane protein on peripheral glia is required to form the blood-nerve barrier in *Drosophila*. *Cell.* **81:** 757-767.

Akazawa, C., Ishibashi, M., Shimizu, C., Nakanishi, S. and Kageyama, R. (1995). A mammalian helix-loop-helix factor structurally related to the product of *Drosophila* proneural gene atonal is a positive transcriptional regulator expressed in the developing nervous system. *J. Biological Chemistry.* **270:** 8730-8738.

Allendoerfer, K. L. and Shatz, C. J. (1994). The subplate: a transient neocortical structure: its role in the development of connections between thalamus and cortex. *Annu. Rev. Neurosci.* **17:** 185-218.

Armson, P., Bennet, M. R. and Raju, T. (1987). Retinal ganglion cell survival and neurite regeneration requirements: the change from Muller cell dependence to superior colliculi dependence during development. *Dev. Brain Res.* **32:** 207-216.

Artavanis-Tsakonas, S., Matsuno, K. and Fortini, M. E. (1995). Notch signaling. *Science.* **268:** 225-232.

Ashley, J. A. and Katz, F. N. (1994). Competition and position-dependent targeting in the development of the *Drosophila* R7 visual projections. *Dev.* **120:** 1537-1547.

Bähr, M. and Bonhoeffer, F. (1994). Perspectives on axonal regeneration in the mammalian CNS. *TINS.* **17:** 473-479.

Baier, H. and Bonhoeffer, F. (1992). Axon guidance by gradients of a target derived component. *Science.* **255:** 472-475.

Baier, H. and Bonhoeffer, F. (1994). Attractive axon guidance molecules. *Science.* **265:** 1541-1542.

Baker, N. E. (1987). Molecular cloning of sequences from wingless, a segment polarity gene in *Drosophila*: the spatial distribution of a transcript in embryos. *EMBO J.* **6:** 1765-1773.

Baker, N. E. and Zitron, A. E. (1995). *Drosophila* eye development: *Notch* and *Delta* amplify a neurogenic pattern conferred on the morphogenetic furrow by *scabrous*. *Mech. of Dev.* **49:** 173-189.

Barres, B. A. and Raff, M. C. (1993). Proliferation of oligodendrocyte precursor cells depends on electrical activity in axons. *Nature.* **361:** 258-260.

Barres, B. A. and Raff, M. C. (1994). Control of oligodendrocyte number in the developing rat optic nerve. *Neuron.* **12:** 935-942.

Bastiani, M. J., du Lac, S. and Goodman, C. S. (1986). Guidance of neuronal growth cones in the grasshopper embryo. I. Recognition of a specific axonal pathway by the pCC neuron. *J. Neurosci.* **6:** 3518-3531.

Bastiani, M. J. and Goodman, C. S. (1986). Guidance of neuronal growth cones in the grasshopper embryo. III. Recognition of specific glial pathways. *J. Neurosci.* **6:** 3542-3551.

Bate, C. M. (1976). Pioneer neurons in an insect embryo. *Nature.* **260:** 54-56.

Bentley, D. and Keshishian, H. (1982). Pioneer neurons and pathways in insect appendages. *TINS.* **5:** 354-358.

Bentley, D. and Caudy, M. (1983). Pioneer axons lose directed growth after selective killing of guidepost cells. *Nature.* **304:** 62-65.

Bermudo, M. D., Carmena, A. and Jimenez, F. (1994). Neurogenic genes control gene expression at the transcriptional level in early neurogenesis and in mesoderm specification. *Dev.* **121:** 219-224.

Bhattacharyya, A., Brackenbury, R., and Ratner, N. (1994). Axons arrest the migration of Schwann cell precursors. *Dev.* **120:** 1411-1420.

Billin, A. N., Cockerill, K. A., and Poole, S. J. (1991). Isolation of a family of *Drosophila* POU domain genes expressed in early development. *Mech. of Dev.* **34:** 75-84.

Blair, S. and Palka, J. (1985). Axon guidance in cultured wing discs and disc fragments. *Dev. Biol.* **108:** 411-419.

Blair, S. S., Murray, M. A., and Palka, J. (1987). The guidance of axons from transplanted neurons through aneural *Drosophila* wings. **7:** 4165-4175.

Blochlinger, K., Bodmer, R., Jack, J., Jan, L. Y., and Jan, Y-N. (1988). Primary structure and expression of a product from *cut*, a locus involved in specifying sensory organ identity in *Drosophila*. *Nature*. **333:** 629-635.

Blochlinger, K., Jan, L. Y., and Jan, Y-N. (1991). Transformation of sensory organ identity by ectopic expression of Cut in *Drosophila*. *Genes and Dev.* **5:** 1124-1135.

Bodmer, R., Barbel, S., Shepherd, S., Jack, J., Jan, L. Y., and Jan, Y-N. (1987). Transformation of sensory organs by mutations of the *cut* locus of *D. melanogaster*. *Cell.* **51:** 293-307.

Bodmer, R., Carretto, R., and Jan, Y-N. (1989). Neurogenesis of the peripheral nervous system in *Drosophila* embryos: DNA replication patterns and cell lineages. *Neuron*. **3:** 21-32.

Bossing, T. and Technau, G. M. (1994). The fate of the CNS midline progenitors in *Drosophila* as revealed by a new method for single cell labeling. *Dev.* **120:** 1895-1906.

Boyan, G., Therianos, S., Williams, J. L. D., and Reichert, H. (1995). Axiogenesis in the embryonic brain of the grasshopper *Schistocerca gregaria*: an identified cell analysis of early brain development. *Dev.* **121:** 75-86.

Bray, G. M., Raminsky, M., and Aguayo, A. J. (1981). Interactions between axons and their sheath cells. *Ann. Rev. Neurosci.* **4:** 127-162.

Brewster, R. and Bodmer, R. (1995). Origin and specification of type II sensory neurons in *Drosophila*. *Dev.* **121:** 2923-2936.

Bronner-Fraser, M. and Fraser, S. E. (1989). Developmental potential of avian trunk neural crest cells *in situ*. *Neuron*. **3:** 755-766.

Brown, N. L., Sattler, C.A., Paddock, S. W., and Carroll, S. B. (1995). Hairy and EMC negatively regulate morphogenetic furrow progression in the Drosophila eye. *Cell.* **80:** 879-887.

Brown, J. L. and Wu, C. (1993). Repression of *Drosophila* pair-rule segmentation genes by ectopic expression of *tramtrack*. *Dev.* **113:** 45-58.

Brunner, A., Twardzik, T. and Schneuwly, S. (1994). The *Drosophila giant lens* gene plays a dual role in eye and optic lobe development: inhibition of the differentiation of ommatidial cells and interference in photoreceptor axon guidance. *Mech of Dev.* **48:** 175-185.

Buchanan, R. L. and Benzer, S. (1993). Defective glia in the *Drosophila* brain degeneration mutant *drop-dead*. *Neuron*. **10:** 839-850.

Buenzow, D. E. and Holmgren, R. (1995). Expression of the *Drosophila gooseberry* locus defines a subset of neuroblast lineages in the CNS. *Dev. Biol.* **170**: 338-349.

Calof, A. (1995). Intrinsic and extrinsic factors regulating vertebrate neurogenesis. *Curr. Op. Neur.* **5:** 19-27.

Campos, A. R., Fischbach, K. F. and Steller, H. (1992). Survival of photoreceptor neurons in the compound eye of *Drosophila* depends on connections with the optic ganglia. *Dev.* **114:** 355-366.

Campos-Ortega, J. A. (1994). Genetic mechanisms of early neurogenesis in *Drosophila melanogaster*. *J. Physiology*, Paris. **88:** 111-122.

Campuzano, S. and Modolell, J. (1992) Patterning of the *Drosophila* nervous system: the *achaete-scute* gene complex. *TIGS*. **8:** 202-208.

Cattaneo, E. and McKay, R. (1990). Proliferation and differentiation of neuronal stem cells regulated by nerve growth factor. *Nature*. **347:** 762-765.

Cheng, H-J. and Flanagan, J. G. (1994). Identification and cloning of ELF-1, a developmentally expressed ligand for the Mek4 and Sek receptor tyrosine kinases. Cell. **79:** 157-168.

Cheng, H-J., Nakamoto, M., Bergemann, A. D., and Flanagan, J. G. (1995). Complementary gradients in expression and binding of ELF-1 and Mek4 in development of the topographic retinotectal projection map. *Cell*. **82:** 371-381.

Chenn, A. and McConnell, S. K. (1995). Cleavage orientation and the asymmetric inheritance of Notch1 immunoreactivity in mammalian neurogenesis. *Cell*. **82:** 631-641.

Cheyette, B. N. R., Green, P. J., Martin, K., Garren, H., Hartenstein, V., and Zipursky, S. L. (1994). The *Drosophila sine oculis* locus encodes a homeodomain-containing protein required for the development of the entire visual system. *Neuron*. **12:** 977-996.

Chiba, A., Snow, P., Keshishian, H., Hotta, Y. (1995). Fasiclin III as a synaptic target recognition molecule in *Drosophila*. *Nature*. **374:** 166-168.

Chitnis, A., Henrique, D., Lewis, J., Ish-Horowicz, D., and Kintner, C. (1995). Primary neurogenesis in *Xenopus* embryos regulated by a homologue of the *Drosophila* neurogenic gene *Delta*. *Nature*. **375:** 761-766.

Choi, K-W., and Benzer, S. (1994). Migration of glia along photoreceptor axons in the developing *Drosophila* eye disc. *Neuron*. **12:** 423-431.

Chu-LaGraff, Q., and Doe, C. Q. (1993). Neuroblast specification regulated by wingless in the *Drosophila* CNS. *Science*. **261:** 1594-1597.

Coffman, C., Harris, W., Kintner, C. (1990). Xotch, the *Xenopus* homologue of *Drosophila* Notch. *Science*. **249:** 1438-1441.

Coffman, C. A., Skoglund, P., Harris, W. A., Kintner, C. R. (1993). Expression of an extracellular deletion of Xotch diverts cell fate in *Xenopus* embryos. *Cell*. **73:** 659-671.

Colamarino, S. A. and Tessier-Lavigne, M. (1995). The axonal chemoattractant netrin-1 is also a chemorepellent for trochlear motor axons. *Cell*. **81:** 621-629.

Colamarino, S. A. and Tessier-Lavigne, M. (1995). The role of the floor plate in axon guidance. *Annu. Rev. Neurosci*. **18:** 497-529.

Condron, B. G. and Zinn, K. (1994). The grasshopper median neuroblast is a multipotent progenitor cell that generates glia and neurons in distinct temporal phases. *J. Neuroscience*. **14:** 5766-5777.

Condron, B. G., Patel, N., and Zinn, K. (1994). *engrailed* controls glial/neuronal fate decisions at the midline of the central nervous system. *Neuron*. **13:** 541-554.

Copenhaver, P. F. and Taghert, P. H. (1990). Neurogenesis in the insect enteric nervous system: generation of pre-migratory neurons from an epithelial placode. *Dev*. **109:** 17-28.

Cox, E. C., Muller, B., Bonhoeffer, F. (1990). Axonal guidance in the chick visual system: posterior tectal membranes induce collapse of growth cones from the temporal retina. *Neuron*. **4:** 31-37.

Crossin, K. L., Hoffman, S., Tang, S. S., and Edelman, G. M. (1989). Cytotactin and its proteoglycan ligand mark structural and functional boundaries in somatosensory cortex of the early postnatal mouse. *Dev. Biol*. **136:** 381-392.

Cubas, P. and Modolell, J. (1992). The *extramacrochaete* gene provides information for sensory organ patterning. *EMBO J.* **11:** 3385-3393.

Cui, X. and Doe, C. Q. (1992). *ming* is expressed in neuroblast sublineages and regulates gene expression in the *Drosophila* CNS. *Dev.* **116:** 943-952.

Currie, D. A. and Bate, M. (1995). Innervation is essential for the development and differentiation of a sex-specific adult muscle in *Drosophila melanogaster.* *Dev.* **121:** 2549-2557.

Dambly-Chaudiere, C., Jamet, E., Burri, M., Bopp, D., Basler, K., Hafen, E., Dumont, N., Spielman, P., Ghysen, A., and Noll, M. (1992). The paired box gene *pox-neuro*: a determinant of the poly-innervated sense organs in *Drosophila*. *Cell.* **69:** 159-172.

Datta, S. and Kankel, D. R. (1992). *l(1)trol* and *l(1)devl*, loci affecting the development of the adult central nervous system in *Drosophila melanogaster.* *Genetics.* **130:** 523-537.

Datta, S. (1995) Control of proliferation activation in quiescent neuroblasts of the *Drosophila* CNS. *Dev.* **121:** 1173-1182.

DeHamer, M. K., Geuvara, J., L., Hannon, K., Olwin, B. B., and Calof, A. L. (1994). Genesis of olfactory receptor neurons *in vitro*: regulation of progenitor cell divisions by fibroblast growth factors. *Neuron.* **13:** 1083-1097.

de la Concha, A., Dietrich, U., Weigel, D., and Campos-Ortega, J. A. (1988). Functional interactions of neurogenic genes of *Drosophila melanogaster.* *Genetics.* **118:** 499-508.

Dick, T., Yang, X., Yeo, S., and Chia, W. (1991). Two closely linked *Drosophila* POU domain genes are expressed in neuroblasts and sensory elements. *PNAS.* **88:** 7645-7649.

Dickson, B. J., Dominguez, M., van der Straten, A., and Hafen E. (1995). Control of *Drosophila* photoreceptor cell fates by *phyllopod*, a novel nuclear protein acting downstream of the raf kinase. *Cell.* **80:** 453-462.

Dodd, J. and Schuchardt, A. (1995). Axon guidance: a compelling case for repelling growth cones. *Cell.* **81:** 471-474.

Doe, C. Q. and Goodman, C. S. (1985a). Early events in insect neurogenesis: developmental and segmental differences in the pattern of neuronal precursor cells. *Dev. Biol.* **111**: 193-205.

Doe, C. Q. and Goodman, C. S. (1985b). Early events in insect neurogenesis: the role of cell interactions and cell lineage in the determination of neuronal precursor cells. *Dev. Bio.* **111:** 206-219.

Doe, C. Q., Hiromi, Y., Gehring, W. J., and Goodman, C. S. (1988a). Expression and function of the segmentation gene *fushi tarazu* during *Drosophila* neurogenesis. *Science.* **239:** 170-175.

Doe, C. Q., Smouse, D., and Goodman, C. S. (1988b). Control of neuronal fate by the *Drosophila* segmentation gene, *even-skipped. Nature.* **333:** 376-378.

Doe, C. Q. (1992). Molecular markers for identified neuroblasts and ganglion mother cells in the *Drosophila* CNS. *Dev.* **116:** 855-863.

Dong, Z., Brennan, A., Liu, N., Yarden, Y., Lefkowitz, G., Mirsky, R., and Jessen, K. R. (1995). Neu differentiation factor is a neuron-glia signal and regulates survival, proliferation, and maturation of rat schwann cell precursors. *Neuron.* **15:** 585-596.

Drescher, U., Kremoser, C., Handwerker, C., Loschinger, J., Noda, M., and Bonhoeffer, F. (1995). *In vitro* guidance of retinal ganglion cell axons by RAGS, a 25 kDa tectal protein related to ligands for Eph receptor tyrosine kinases. *Cell.* **82:** 359-370.

Duffy, J. B., Kania, M. A., and Gergen, J. P. (1991). Expression and function of the *Drosophila* gene *runt* in early stages of neural development. *Dev.* **113:** 1223-1230.

du Lac, S., Bastiani, M. J., and Goodman, C. S. (1986). Guidance of neuronal growth cones in the grasshopper embryo. II. Recognition of a specific axonal pathway by the aCC neuron. *J. Neurosci.* **6:** 3532-3551.

Duus, K. M., Welshons, W. J. and Girton, J. R. (1992). *Blackpatch,* a neural degeneration mutation that interacts with the *Notch* locus in *Drosophila. Dev. Biol.* **151:** 34-47.

Ebens, A. J., Garren, H., Cheyette, B. N. R., and Zipursky, S. L. (1993). The Drosophila *anachronism* locus: a glycoprotein secreted by glia inhibits neuroblast proliferation. *Cell.* **74:** 15-28.

Edmondson, J. C., Liem, R. K. H., Kuster, J. E., and Hatten, M. E. (1988). Astrotactin: a novel neuronal cell surface antigen that mediates neuron-astroglial interactions in cerebellar microcultures. *JCB.* **106:** 505-517.

Elkins, T., Hortsch, M., Bieber, A. J., Snow, P. M., and Goodman, C. S. (1990a). *Drosophila* fasiclin I is a homophilic adhesion molecule that along with fas III can mediate cell sorting. *J. Cell. Biol.* **110:** 1825-1832.

Elkins, T., Zinn, K., McAllister, L., Hoffman, F. M. and Goodman, C. S. (1990b). Genetic analysis of a Drosophila neural cell adhesion molecule: interaction of fasiclin I and Ableson tyrosine kinase mutations. *Cell.* **60:** 565-575.

Ernfos, P., Lee, K-F., Kucera, J., and Jaenisch, R. (1994). Lack of neurotrophin-3 leads to deficiencies in the peripheral nervous system and loss of proprioceptive afferents. *Cell.* **77**: 503-512.

Ewer, J., Frisch, B., Hamblen-Coyle, M. J., Rosbash, M. and Hall, J. C. (1992). Expression of the period clock gene within different cell types in the brain of *Drosophila* adults and mosaic analysis of these cells' influence on circadian behavioral rhythms. *J. Neurosci.* **12:** 3321-3349.

Faissner, A. and Steindler, D. (1995). Boundaries and inhibitory molecules in developing neural tissues. *Glia.* **13:** 233-254.

Fan, J. and Raper, J. A. (1995). Localized collapsing cues can steer growth cones without inducing their full collapse. *Neuron.* **14:** 263-274.

Fehon, R. G., Kooh, P. J., Rebay, I., Regan, C. L., Xu, T., Muskavitch, M .A., and Artavanis-Tsakonas, S. (1990). Molecular interactions between the protein products of the neurogenic loci Notch and Delta, two EGF-homologous genes in *Drosophila. Cell.* **61:** 523-534.

Ferreiro, B., Skoglund, P., Bailey, A., Dorsky, R., Harris, W. A. (1992). XASH-1, a *Xenopus* homologue of achaete-scute: a proneural gene in anterior regions of the vertebrate CNS. *Mech. of Dev.* **40:** 25-36.

Fischbach, K. F. (1983). Neural cell types surviving congenital sensory degeneration in the optic lobes of *Drosophila melanogaster. Dev. Biol.* **95:** 1-18.

Fischbach, K. F. and Technau, G. (1984). Cell degeneration in the developing optic lobes of the *sine oculis* and *small-optic-lobes* mutants of *Drosophila melanogaster. Dev. Biol.* **104:** 219-239.

Fishell, G., and Hatten, M. E. (1991). Astrotactin provides a receptor system for glial-guided neuronal migration. *Dev.* **113:** 755-765.

Fortini, M. E. and Artavanis-Tsakonas, S. (1994). The Suppressor of Hairless protein participates in Notch receptor signaling. *Cell.* **79:** 273-289.

Frank, E. and Sanes, J. R. (1991). Lineage of neurons and glia in chick dorsal root ganglia: analysis *in vivo* with a recombinant retrovirus. *Dev.* **111:** 895-908.

Furukawa, T., Maruyama, S., Kawaichi, m. and Honjo, T. (1992). The *Drosophila* homologue of the immunoglobin recombination signal binding protein regulates peripheral nervous system development. *Cell.* **69:** 1191-1197.

Gage, F. H., Ray, J., and Fisher, L. J. (1995). Isolation, characterization, and use of stem cells from the CNS. *Annu. Rev. Neurosci.* **18:** 159-192.

Galileo, D. S., Gray, G. E., Owens, G. C., Majors, J., and Sanes, J. R. (1990). Neurons and glia arise from a common progenitor in chick optic tectum: demonstration with two retroviruses and cell type-specific antibodies. *PNAS.* **87:** 458-462.

Gao, W-Q., Heintz, N., and Hatten, M. E. (1991). Cerebellar granule cell neurogenesis is regulated by cell-cell interactions *in vitro. Neuron.* **6:** 705-715.

Gao, W-Q., and Hatten, M. E. (1994). Immortalizing oncogenes subvert the establishment of granule cell identity in the developing cerebellum. *Dev.* **120:** 1059-1070.

Garrell, J. and Modolell, J. (1990). The *Drosophila extramacrochaete* locus, an antagonist of proneural genes that, like these genes, encodes a helix-loop-helix protein. *Cell.* **61:** 39-48.

Garrity, P. A. and Zipursky, S. L. (1995). Neuronal target recognition. *Cell.* **83:** 177-185.

Gasser, U. E. and Hatten, M. E. (1990). Neuron-glia interactions of rat hippocampal cells in vitro: glial guided neuronal migration and neuronal regulation of glial differentiation. *J. Neuroscience.* **10:** 1276-1285.

Ghosh, A., Antonini, A., McConnell, S. K. and Shatz, C. J. (1990). Requirement for subplate neurons in the formation of thalamocortical connections. *Nature.* **347:** 179-181.

Ghysen, A., Dambly-Chaudiere, C., Jan, L.Y., and Jan, Y-N. (1993). Cell interactions and gene interactions in peripheral neurogenesis. *Genes and Dev.* **7:** 723-733.

Giangrande, A., Murray, M. A., and Palka, J. (1993). Development and organization of glial cells in the peripheral nervous system of *Drosophila melanogaster. Dev.* **117:** 895-904.

Giangrande, A. (1994). Glia in the fly wing are clonally related to epithelial cells and use the nerve as a pathway for migration. *Dev.* **120:** 523-534.

Giangrande, A. (1995). Proneural genes influence gliogenesis in *Drosophila. Dev.* **121:** 429-438.

Godement, P., Salaun, J., and Mason, C. A. (1990). Retinal axon pathfinding in the optic chiasm: divergence of crossed and uncrossed fibers. *Neuron.* **5:** 173-186.

Gong, Q. and Shiplet, M. T. (1995). Evidence that pioneer olfactory axons regulate telencephalon cell cycle kinetics to induce the formation of the olfactory bulb. *Neuron.* **14:** 91-101.

Gonzalez, F., Romani, S., Cubas, P., Modolell, J., and Campuzano, S. (1989). Molecular analysis of the *asense* gene, a member of the *achaete-scute complex* of *Drosophila melanogaster,* and its novel role in optic lobe development. *EMBO J.* **8:** 3553-3562.

Gonzalez, M. d. L., Malemud, C. J., and Silver, J. (1993). Role of astroglial extracellular matrix in the formation of rat olfactory bulb glomeruli. *Exp. Neurology.* **123:** 91-105.

Goodman, C. S. and Shatz, C. J. (1993). Developmental mechanisms that generate precise patterns of neuronal connectivity. *Neuron.* **10:** 77-98.

Gorczyca, M. G., Phillis, R. W., and Budnik, V. (1994). The role of *tinman,* a mesodermal cell fate gene, in axon pathfinding during the development of the transverse nerve in *Drosophila. Dev.* **120:** 2143-2152.

Goriely, A., Dumont, N., Dambly-Chaudiere, C., and Ghysen, A. (1991). The determination of sense organs in *Drosophila:* effect of the neurogenic mutations in the embryo. *Dev.* **113:** 1395-1404.

Gotz, M., Novak, N., Bastmeyer, M., and Bolz, J. (1992). Membrane-bound molecules in rat cerebral cortex regulate thalamic innervation. *Dev.* **116:** 507-519.

Green, P., Hartenstein, V., and Younossi-Hartenstein, A. (1993). The embryonic development of the *Drosophila* visual system. *Cell Tissue Res.* **273:** 583-598.

Grenningloh, G. and Goodman, C. S. (1992). Pathway recognition by neuronal growth cones: genetic analysis of neural cell adhesion molecules in *Drosophila*. *Curr. Op. Neurobio.* **2:** 42-47.

Grenningloh. G., Rehm, E. J., and Goodman, C. S. (1991). Genetic analysis of growth cone guidance in *Drosophila*: *fasiclin II* functions as a neuronal recognition molecule. *Cell.* **67:** 45-57.

Guillemont, F. and Joyner, A. L. (1993). Dynamic expression of the murine *achaete-scute* homologue MASH-1 in the developing nervous system. *Mech of Dev.* **42:** 171-185.

Guillemont, F., Lo, L-C., Johnson, J. E., Auerbach, A., Anderson, D. J., and Joyner, A. L. (1993). Mammalian *achaete-scute* homolog 1 is required for the early development of olfactory and autonomic neurons. *Cell.* **75:** 463-476.

Guo, M., Bier, E., Jan, L. Y. and Jan Y-N. (1995). *tramtrack* acts downstream of *numb* to specify distinct daughter cell fates during asymmetric cell divisions in the *Drosophila* PNS. *Cell.* **14:** 913-925.

Guthrie, S. and Pini, A. (1995). Chemorepulsion of developing motor axons by the floor plate. *Neuron.* **14:** 1117-1130.

Halder, G., Callaerts, P. and Gehring, W. J. (1995). Induction of ectopic eyes by targeted expression of the eyeless gene. *Science.* **267:** 1788-1792.

Hall, A. K. and Landis, S. C. (1991). Early commitment of precursor cells from the rat superior cervical ganglion to neuronal or non-neuronal fates. *Neuron.* **6:** 741-752.

Hamburger, V. and Levi-Montalcini, R. (1949). Proliferation, differentiation, and degeneration in the spinal ganglia of the chick embryo under normal and experimental conditions. *J. Expl. Zool.* **111:** 457-501.

Harrison, S. and Travers, A. (1990). The *tramtrack* gene encodes a *Drosophila* finger protein that interacts with the *ftz* transcriptional regulatory region and shows a novel embryonic expression pattern. *EMBO J.* **9:** 207-216.

Hartenstein, V. and Campos-Ortega, J. A. (1984). Early neurogenesis in wild type *Drosophila melanogaster*. *Roux's Arch. Dev. Biol.* **193:** 308-325.

Hartenstein, V. and Campos-Ortega, J. A. (1986). The peripheral nervous system of mutants of early neurogenesis in *Drosophila melanogaster*. *Roux's Arch. Dev. Biol.* **195:** 210-221.

Hartenstein, V. and Posakony, J. W. (1990). A dual function of the *Notch* gene in *Drosophila* sensillum development. *Dev. Biol.* **142:** 13-30.

Hatten, M. E. and Heintz, N. (1995). Mechanisms of neural patterning and specification in the developing cerebellum. *Annu. Rev. Neurosci.* **18:** 385-408.

Heberlein, U. and Rubin, G. M. (1991). *Star* is required in a subset of photoreceptor cells in the developing *Drosophila* retina and displays dosage sensitive interactions with *rough*. *Dev. Biol.* **144:** 353-361.

Heberlein, U., Wolff, T. and Rubin, G. M. (1993). The TGFβ1 homolog *dpp* and the segment polarity gene *hedgehog* are required for propagation of a morphogenetic wave in the *Drosophila* retina. *Cell.* **75:** 913-926.

Heberlein, U. and Moses, K. (1995). Mechanisms of *Drosophila* retinal morphogenesis: the virtues of being progressive. *Cell.* **81:** 987-990.

Henrique, D., Adam, J., Myat, A., Chitnis, A. Lewis, J., and Ish-Horowicz, D. (1995). Expression of a *Delta* homologue in prospective neurons in the chick. *Nature.* **375:** 787-790.

Hinz, U., Giebel, B., and Campos-Ortega, J. A. (1994). The basic helix-loop-helix domain of *Drosophila* lethal of scute protein is sufficient for proneural function and activates neurogenic genes. *Cell.* **76:** 77-87.

Hofbauer, A. and Campos-Ortega, J. A. (1990). Proliferation pattern and early differentiation of the optic lobes in *Drosophila melanogaster*. *Roux Arch. Dev. Biol.* **198:** 264-274.

Hoppe, P. E. and Greenspan, R. J. (1986). Local function of the *Notch* gene for embryonic ectodermal pathway choice in *Drosophila*. *Cell.* **46:** 773-783.

Hosoya, T., Takizawa, K., Nitta, K, and Hotta, Y. (1995). *glial cells missing*: a binary switch between neuronal and glial differentiation in *Drosophila*. *Cell.* **82:** 1025-1036.

Ishii, N., Wadsworth, W. G., Stern, B. D., Culotti, J. G. and Hedgecock, E. M. (1992). UNC-6, a laminin related protein, guides cells and pioneer axon migration in *C. elegans*. *Neuron.* **9:** 873-881.

Ito, K. and Hotta, Y. (1992). Proliferation pattern of postembryonic neuroblasts in the brain of *Drosophila melanogaster*. *Dev. Biol.* **149:** 134-148.

Ito, K., Urban, J. and Technau, G. M. (1995). Distribution, classification, and development of *Drosophila* glial cells in the late embryonic and early larval ventral nerve cord. *Roux's Arch. Dev. Biol.* **204:** 284-307.

Jacobs, J. R. and Goodman, C. S. (1989a). Embryonic development of axon pathways in the *Drosophila* CNS. I. A glial scaffold appears before the first growth cones. *J. Neurosci.* **9:** 2402-2411.

Jacobs, J. R. and Goodman, C. S. (1989b). Embryonic development of axon pathways in the *Drosophila* CNS. II. Behavior of pioneer growth cones. *J. Neurosci.* **9:** 2412-2422.

Jacobs, J. R., Hiromi, Y., Patel, N., and Goodman, C.S. (1989). Lineage, migration, and morphogenesis of longitudinal glia in the *Drosophila* CNS as revealed by a molecular lineage marker. *Neuron.* **2:** 1625-1631.

Jacobs, J. R. (1993). Perturbed glial scaffold formation precedes axon tract malformation in *Drosophila* mutants. *J. Neurobiology.* **24:** 611-626.

Jan, Y-N., Ghysen, A., Christoph, I., Barbel, S., and Jan, L. Y. (1985). Formation of neuronal pathways in the imaginal discs of *Drosophila*. *J. Neurosci.* **5:** 2453-2464.

Jan, Y-N. and Jan, L. (1994). Neuronal cell fate specification in *Drosophila*. *Curr. Op. Neurobio.* **4:** 8-13.

Jarman, A. P., Grau, Y., Jan, L. Y., and Jan, Y-N. (1993). *atonal* is a proneural gene that directs chordotonal organ formation in the *Drosophila* peripheral nervous system. *Cell.* **73:** 1307-1321.

Jarman, A. P., Grell, E. H., Ackerman, L., Jan, L. Y. and Jan, Y-N. (1994). *atonal* is the proneural gene for *Drosophila* photoreceptors. *Nature.* **369:** 398-400.

Jarriault, S., Brou, C., Logeat, F., Schroeter, E. H., Kopan, R., and Israel, A. (1995). Signaling downstream of activated mammalian *Notch*. *Nature.* **377:** 355-358.

Jennings, B., Preiss, A., Delidakis, C., and Bray, S. (1994). The *Notch* signaling pathway is required for *Enhancer of split* bHLH protein expression in the *Drosophila* embryo. *Dev.* **120:** 3537-3548.

Jessen, K. R. and Mirsky, R. (1992). Schwann cells: early lineage, regulation of proliferation and control of myelin formation. *Curr. Op. Neurobio.* **2:** 575-581.

Jessen, K. R., Brennan, A., Morgan, L., Mirsky, R., Kent, A., Hashimoto, Y., and Gavrilovic, J. (1994). The schwann cell precursors and its fate: a study of cell death and differentiation during gliogenesis in rat embryonic nerves. *Neuron.* **12:** 509-527.

Jimenez, F. and Campos-Ortega, J. A. (1987). Genes in subdivision 1B of the *Drosophila melanogaster* X chromosome and their influence on neural development. *J. Neurogenetics.* **4:** 179-200.

Jimenez, F. and Campos-Ortega, J. A. (1990). Defective neuroblast commitment in mutants of the achaete-scute complex and adjacent genes of *Drosophila melanogaster. Neuron.* **5:** 81-89.

Jimenez, F., Martin-Morris, L. E., Velasco, L., Chu, H., Sierra, J., Rosen, D. R., and White, K. (1995). *vnd*, a gene required for early neurogenesis of *Drosophila* encodes a homeodomain protein. *EMBO J.* **14:** 3487-3495.

Johnson, J. E., Birren, S. J., and Anderson, D. J. (1990). Two rat homologues of *Drosophila achaete-scute* specifically expressed in neuronal precursors. *Nature.* **346:** 858-861.

Johnston, A. R. and Gooday, D. J. (1991). *Xenopus* temporal retinal neurites collapse on contact with glial cells from caudal tectum *in vitro. Dev.* **113:** 409-417.

Jones, B. W., Fetter, R. D., Tear, G., and Goodman, C. S. (1995). *glial cells missing*: a genetic switch that controls glial versus neuronal fate. *Cell.* **82:** 1013-1023.

Kalcheim, C., Carmeli, C., and Rosenthal, A. (1992). Neurotrophin 3 is a mitogen for cultured neural crest cells. *PNAS.* **89:** 1661-1665.

Kankel, D. R., Ferrus, A., Garen, S. H., Harte, P. J., and Lewis, P. E. (1980). The structure and development of the nervous system. In *The Genetics and Biology of Drosophila.* (eds. M. Ashburner and T. R. F. Wright). pp. 295-363. Academic Press: New York.

Kaphingst, K. and Kunes, S. K. (1994). Pattern formation in the visual centers of the *Drosophila* brain: *wingless* acts via *decapentaplegic* to specify the dorsoventral axis. *Cell.* **78:** 437-448.

Kennedy, T. E., Serafini, T., de la Torre, J. and Tessier-Lavigne, M. (1994). Netrins are diffusible chemotrophic factors for commissural axons in the embryonic spinal cord. *Cell.* **78:** 425-435.

Kennedy, T. E., and Tessier-Lavigne. (1995). Guidance and induction of branch formation in developing axons by target derived diffusible factors. *Curr. Op. Neurobio.* **5:** 83-90.

Kessler, D. S. and Melton, D. A. (1994). Vertebrate embryonic induction: mesodermal and neural patterning. *Science.* **266:** 596-604.

Keynes, R. J. and Cook, G. M. W. (1995). Repulsive and inhibitory signals. *Curr. Op. Neurobio.* **5:** 75-82.

Kimmel, B. E., Heberlein, U. and Rubin, G. M. (1990). The homeo domain protein *rough* is expressed in a subset of cells in the developing *Drosophila* eye where it can specify photoreceptor cell subtype. *Genes and Dev.* **4:** 712-727.

Klaes, A., Menne, T., Stollewerk, A., Schols, H., and Klämbt, C. (1994). The ETS transcription factors encoded by the *Drosophila* gene *pointed* direct glial cell differentiation in the embryonic CNS. *Cell.* **78:** 149-160.

Klämbt, C., Jacobs, R. J., and Goodman, C. S. (1991). The midline of the *Drosophila* CNS: a model for the genetic analysis of cell fate, cell migration, and growth cone guidance. *Cell.* **64:** 801-815.

Klämbt, C. (1993). The *Drosophila* gene pointed encodes two ETS-like proteins, which are involved in the development of the midline glial cells. *Dev.* **117:** 163-176.

Knust, E., Schrous, H., Grawe, F., and Campos-Ortega, J. A. (1992). Seven genes of the *E(spl)* complex of *Drosophila melanogaster* encode helix-loop-helix proteins. *Genetics.* **132:** 505-518.

Knust, E. (1994). Cell fate choice during early neurogenesis in *Drosophila melanogaster. Perspectives on Developmental Neurobiology.* **2:** 141-149.

Kolodkin, A. L., Matthes, D. J., O'Connor, T. P., Patel, N. H., Admon, A., Bentley, D. and Goodman, C. S. (1992). Fasiclin IV: sequence, expression, and function during growth cone guidance in the grasshopper embryo. *Neuron.* **9:** 831-845.

Kolodkin, A. L., Matthes, D. J., and Goodman, C. S. (1993). The *semaphorin* genes encode a family of transmembrane and secreted growth cone guidance molecules. *Cell.* **75:** 1389-1399.

Kopan, R., Nye, J. S., and Weintraub, H. (1994). The intracellular domain of mouse Notch: a constitutively activated repressor of myogenesis directed at the basic helix-loop-helix region of MyoD. *Dev.* **120:** 2385-2396.

Kramatschek, B. and Campos-Ortega, J. A. (1994). Neuroectodermal transcription of the *Drosophila* neurogenic genes *E (spl)* and HLH-m5 is regulated by proneural genes. *Dev.* **120:** 815-826.

Kretzschmar, D., Brunner, A., Wiersdorff, V., Pflugfelder, G. O., Heisenberg, M. and Schneuwly, S. (1992). *giant lens*, a gene involved in cell determination and axon guidance in the visual system of *Drosophila* melanogaster. *EMBO J.* **11:** 2531-2539.

Kuhn, T. B., Schmidt, M. F., and Kater, S. B. (1995). Laminin and fibronectin guideposts signal sustained but opposite effects to passing growth cones. *Neuron.* **14:** 275-285.

Kunes, S., Wilson, C. and Steller, H. (1993). Independent guidance of retinal axons in the developing visual system of *Drosophila. J. Neurosci.* **13:** 752-767.

Kunes, S. K. and Steller, H. (1993). Topography in the *Drosophila* visual system. *Curr. Op. Neurobio.* **3:** 53-59.

Lander, A. D. (1990). Mechanisms by which molecules guide axons. *Curr. Op. Cell. Bio.* **2:** 907-913.

Lane, N. J. (1981). Invertebrate neuroglia-junctional structure and development. *J. Exp. Biol.* **95:** 7-33.

Lawrence, P. A. and Green, S. M. (1979). Cell lineage in the developing retina of the *Drosophila. Dev. Biol.* **71:** 142-152.

Le Douarin, N. M. and Smith, J. (1988). Development of the peripheral nervous system from the neural crest. *Ann Rev. Cell Biol.* **4:** 375-404.

Le Douarin, N. M. and Dupin, E. (1992). Cell lineage analysis in neural crest ontogeny. *J. Neurobiology.* **24:** 146-161.

Lee, J. E., Hollenberg, S. M., Snider, L., Turner, D. L., Lipnick, N., and Weintraub, H. (1995). Conversion of *Xenopus* ectoderm into neurons by NeuroD, a basic helix-loop-helix protein. *Science.* **268:** 836-844.

Lefcort, F. and Bentley, D. (1987). Pathfinding by pioneer neurons in isolated, opened and mesoderm-free limb buds of embryonic grasshoppers. *Dev. Biol.* **119:** 466-480.

Lehmann, R., Jimenez, F., Dietrich, U., and Campos-Ortega, J. (1983). On the phenotype and development of mutants of early neurogenesis in *Drosophila melanogaster. Roux. Arch. Dev. Biol.* **192:** 62-74.

Leiber, T., Kidd, S., Alcamo, E., Corbin, V., and Young, M. W. (1993). Antineurogenic phenotypes induced by truncated *Notch* proteins indicate a role in signal transduction and may point to a novel function for *Notch* in nuclei. *Genes and Dev.* **7:** 1949-1965.

Le Lievre, C. S., Schweizer, G. G., Ziller, C. M., and Le Douarin, N. M. (1980). Restrictions of developmental capabilities in neural crest cell derivatives as tested by *in vivo* transplantation experiments. *Dev. Biol.* **77:** 362-378.

Levine, R. B., Morton, D. B., and Restifo, L. L. (1995). Remodeling of the insect nervous system. *Curr. Op. Neurobio.* **5:** 28-35.

Lillien, L. (1994). Neurogenesis in the vertebrate retina. *Perspectives on Developmental Neurobiology.* **2:** 175-182.

Lin, D. M., Fetter, R. D., Kopczynski, C., Grenningloh, G., and Goodman, C. S. (1994). Genetic analysis of *fasiclin II* in *Drosophila*: defasiculation, refeasiculation, and altered fasiculation. *Neuron.* **13:** 1055-1069.

Lin, D. M., Auld, V. J., and Goodman, C. S. (1995). Targeted neuronal cell ablation. *Neuron.* **14:** 707-715.

Lin, L-F. H., Doherty, D. H., Lile, J. D., Bektesh, S., and Collins, F. (1993). GDNF: a glial cell line-derived neurotrpophic factor for midbrain dopaminergic neurons. *Science.* **260:** 1130-1132.

Lipshitz, H. D. and Kankel, D. R. (1985). Specificity of gene action during central nervous system development in *Drosophila melanogaster*: analysis of the *lethal (1) optic ganglion reduced* locus. *Dev. Biol.* **108:** 56-77.

Lo, L-C., Johnson, J. E., Wuenschell, C. W., Saito, T., and Anderson, D. J. (1991). Mammalian *achaete-scute* homolog 1 is transiently expressed by spatially restricted subsets of early neuroepithelial and neural crest cells. *Genes and Dev.* **5:** 1524-1537.

Luo, Y., Raible, D., and Raper, J. A. (1993). Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. *Cell.* **75:** 217-227.

Luo, Y., Shepherd, I., Li, J., Renzi, M. J., Chang, S., and Raper, J. A. (1995). A family of molecules related to collapsin in the embryonic chick nervous system. *Neuron.* **14:** 1131-1140.

Luskin, M. B. (1994). Neuronal cell lineage in the vertebrate central nervous system. *FASEB J.* **8:** 722-730.

Luskin, M. B. and McDermott, K. (1994). Divergent lineages for oligodendrocytes and astrocytes originating in the neonatal forebrain subventricular zone. *Glia.* **11:** 211-226.

Ma, C., Zhou, Y., Beachy, P. A. and Moses, K. (1993). The segment polarity gene *hedgehog* is required for progression of the morphogenetic furrow in the developing eye disc. *Cell.* **75:** 927-938.

Maier, D., Marte, B. M., Schafer, W., Yu, Y., and Preiss, A. (1993). *Drosophila* evolution challenges postulated redundancy in the *E(spl)* gene complex. *PNAS.* **90:** 5464-5468.

Martin, J. H. and Jessell, T. M. (1991). Development as a guide to the regional anatomy of the brain. In *Principles of Neuroscience.* (eds. E. R. Kandel, J. H. Schwartz, and T. M. Jessel). pp. 296-308. New York: Elsevier.

Martin-Bermudo, M. D., Martinez, C., Rodriguez, A., and Jimenez, F. (1991). Distribution and function of the *lethal of scute* gene product during early neurogenesis in *Drosophila. Dev.* **113:** 445-454.

Matthes, D. J., Sink, H., Kolodkin, A., and Goodman, C. S. (1995). Semaphorin II can function as a selective inhibitor of specific synaptic arborizations. *Cell.* **81:** 631-639.

McConnell, S. K. (1989). Subplate neurons pioneer the first axon pathway from the cerebral cortex. *Science.* **245:** 978- 982.

McConnell, S. K. (1992). The control of neuronal identity in the developing cerebral cortex. *Curr. Op. Neurobio.* **2:** 23-27.

McConnell, S. K. and Kaznowski, C. E. (1991). Cell cycle dependence of laminar determination in developing neocortex. *Science.* **254:** 282-286.

Meinertzhage, I. A. (1973). Development of the compound eye and optic lobe of insects. In *Developmental Neurobiology of Arthropods.* (ed. D. Young). pp. 51-104. Cambridge: Cambridge University Press.

Meinertzhagen, I. A. and Hanson, T. E. (1993). The development of the optic lobe. In *The Development of Drosophila melanogaster.* (eds M. Bate and A. Martinez-Arias). pp., 1363-1491. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Menne, T. V. and Klämbt, C. (1994). The formation of commissures in the *Drosophila* CNS depends on the midline cells and on the *Notch* gene. *Dev.* **120:** 123-133.

Messersmith, E. K., Leonardo, E. D., Shatz, C. J., Tessier-Lavigne, M., Goodman, C. S. and Kolodkin, A. L. (1995). Semaphorin III can function as a selective chemorepellent to pattern sensory projections in the spinal cord. *Neuron.* **14:** 949-959.

Mlodzik, M., Baker, N. E., and Rubin, G.M. (1990). Isolation and expression of *scabrous*, a gene regulating neurogenesis in *Drosophila. Genes and Dev.* **4:** 1848-1861.

Morshead, C. M., Reynolds, B. A., Craig, C. G., McBurney, M. W., Staines, W. A., Morassutti, D., Weiss, S., Van der Kooy, D. (1994). Neural stem cells in the adult mammalian forebrain: a relatively quiescent subpopulation of subependymal cells. *Neuron.* **13:** 1071-1082.

Nakato, H., Futch, T. A. and Selleck, S. B. (1995). The *division abnormally delayed*(*dally*) gene ; a putative integral membrane protein required for cell division patterning during post-embryonic development in *Drosophila. Dev.* **121:** 3687-3702.

Noble, M., Fok-Seang, J., Cohen, J. (1984). Glia are a unique substrate for the in vitro growth of central nervous system neurons. *J. Neuroscience.* **4:** 1892-1903.

Norris, C. R. and Kalil, K. (1991). Guidance of callosal axons by radial glia in the developing cerebral cortex. *J. Neuroscience.* **11:** 3481-3492.

Nottebohm, E., Dambly-Chaudiere, C., and Ghysen, A. (1992). Connectivity of chemosensory neurons is controlled by the gene *poxn* in *Drosophila. Nature.* **359:** 829-832.

Nye, J. S., Kopan, R., and Axel, R. (1994). An activated *Notch* suppresses neurogenesis and myogenesis but not gliogenesis in mammalian cells. *Dev.* **120:** 2421-2430.

Ohsako, S., Hyer, J., Panganiban, G., Oliver, I., and Caudy, M. (1994). *hairy* functions as a DNA binding helix-loop-helix repressor of *Drosophila* sensory organ formation. *Genes and Dev.* **8:** 2743-2755.

Pflugfelder, G. O., Roth, H., Poeck, B. Kerscher, S., Schwarz, S., Jonschker, B., and Heisenberg, M. (1992). The *l(1) optomotor-blind* gene of *Drosophila melanogaster* is a major organizer of optic lobe development: isolation and characterization of the gene. *PNAS.* **89:** 1199-1203.

Placzek, M., Tessier-Lavigne, M., Jessell, T., and Dodd, J. (1990). Orientation of commissural axons in vitro in response to a floor plate derived chemoattractant. *Dev.* **110:** 19-30.

Poulson, D. F. (1950). Histogenesis, organogenesis, and differentiation in the embryo of Drosophila melanogaster. In *Biology of Drosophila melanogaster.* (ed. M. Demerec). pp. 168-274. New York: John Wiley and Sons.

Power, M. E. (1943). The brain of *Drosophila melanogaster. J. Exp. Zool.* **94:** 33-71.

Price, J., Turner, D., and Cepko, C. L. (1987). Lineage analysis in the vertebrate nervous system by retrovirus mediated gene transfer. *PNAS.* **84:** 156-160.

Prokop, A. and Technau, G. M. (1991). The origin of postembryonic neuroblasts in the ventral nerve cord of *Drosophila melanogaster. Dev.* **111:** 79-88.

Puschel, A. W., Adams, R. H., and Betz, H. (1995). Murine semaphorin D/collapsin is a member of a diverse gene family and creates domains inhibitory for axonal extension. *Neuron.* **14:** 941-948.

Purves, D. and Lichtman, J. W. (1985). *Principles of Neural Development.* Sunderland, Massaachusetts: Sinauer Associates, Inc.

Quiring, R., Walldorf, U., Kloter, U. and Gehring, W. J. (1994). Homology of the *eyeless* gene of *Drosophila* to the Small eye gene in mice and aniridia in humans. *Sciene.* **265:** 785-789.

Raff, M. C., Abney, E. R., Fok-Seang, J. (1985). Reconstitution of a developmental clock *in vitro*: a critical role for astrocytes in the timing of oligodendrocyte differentiation. *Cell.* **42:** 61-69.

Raff, M. C. (1989). Glial cell diversification in the rat optic nerve. *Science.* **243:** 1450-1455.

Ramos, R. G. P., Igloi, G. L., Lichte, B., Baumann, U., Maier, D., Schneider, T., Brandstatter, J. H., Frohlich, A., and Fischbach, K-F. (1993). The *irregular chiasm C-roughest* locus of *Drosophila*, which affects axonal projections and programmed cell death, encodes a novel immunoglobin like protein. *Genes and Dev.* **7:** 2533-2547.

Rao, Y., Vaessin, H., Jan, L. Y., and Jan, Y-N. (1991). Neuroectoderm in *Drosophila* embryos is dependent on the mesoderm for positioning but not for formation. *Genes and Dev.* **5:** 1577-1588.

Rao, Y., Bodmer, R., Jan, L. Y., and Jan, Y-N. (1992). The *big brain* gene of *Drosophila* functions to control the number of neuronal precursors in the peripheral nervous system. *Dev.* **116:** 31-40.

Raper, J. A., Bastiani, M. J., and Goodman, C. S. (1983a). Pathfinding by neuronal growth cones in grasshopper embryos. I. divergent choices made by the growth cones of sibling neurons. *J. Neurosci.* **3:** 20-30.

Raper, J. A., Bastiani, M. J. and Goodman, C. S. (1983b). Pathfinding by neuronal growth cones in grasshopper embryos. *J. Neurosci.* **3:** 31-41.

Ready, D. F., Hanson, T. E. and Benzer, S. (1976). Development of the *Drosophila* retina, a neurocrystalline lattice. *Dev. Biol.* **53:** 217-240.

Reaume, A. G., Conlon, R. A., Zirngibl, R., Yamaguchi, T. P., and Rossant, J. (1992). Expression analysis of a *Notch* homologue in the mouse embryo. *Dev. Biol.* **154:** 377-387.

Reichenbach, A. (1991). Glial K+ Permeability and CNS K+ clearance by diffusion and spatial buffering. In *Glial-Neuronal Interactions.* (ed. N. J. Abbott). pp. 272-286. New York: New York Academy of Sciences.

Reh, T. A. (1987). Cell-specific regulation of neuronal production in the larval frog retina. *J. Neuroscience.* **10:** 3317-3324.

Reynolds, B. A. and Weiss, S. (1992). Generation of neurons and astrocytes from isolated cells of the mammalian central nervous system. *Science.* **255:** 1707-1710.

Rohrer, H. and Thoenen, H. (1987). Relationship between differentiation and terminal mitosis: chick sensory and ciliary neurons differentiate after terminal mitosis of precursor cells, whereas sympathetic neurons continue to divide after differentiation. *J. Neuroscience.* **7:** 3739-3748.

Rushlow, C.A., Hogan, A., Pinchin, S. M., Howe, K.M., Lardelli, M., and Isc-Horowicz, D. (1989). The *Drosophila hairy* protein acts in both segmentation and bristle patterning and shows homology to N-myc. *EMBO J.* **8:** 3095-3103.

Russell, M. (1982). Imaginal discs. In *Handbook of Drosophila Development,* (ed. R. Ransom). pp. 95-121. Elsevier Biomedical: New York.

Rhyu, M. S., Jan, L. Y., and Y-N. Jan. (1994). Asymmetric distribution of numb protein during division of the sensory organ precursor cell confers distinct fates to daughter cells. *Cell.* **76:** 477-491.

Salzberg, A., D'Evelyn, D., Schulze, K. L., Lee, J-K., Strumpf, D., Tsai, L., and Bellen, H. J. (1994). Mutations affecting the pattern of the PNS in *Drosophila* reveal novel aspects of neuronal development. *Neuron.* **13:** 269-287.

Schlue, W-R., Dorner, R., Rempe, L., and Riehl, B. (1991). Glial H+ transport and control of pH. In *Glial-Neuronal Interactions.* (ed. N. J. Abbott). pp. 287-305. New York: New York Academy of Sciences.

Schneider, T., Reiter, C., Eule, E., Bader, B., Lichte, B., Nie, Z., Schimansky, T., Ramos, R. G. P. and Fischbach, K-F. (1995). Restricted expression of the *irreC-rst* protein is required for normal axonal projections of columnar visual neurons. *Neuron.* **15:** 259-271.

Schweisguth, F. and Posakony, J. W. (1992). *Suppressor of Hairless,* the *Drosophila* homologue of the mouse recombination signal binding protein gene, controls sensory organ cell fates. *Cell.* **69:** 1199-1212.

Schweisguth, F. and Posakony, J. W. (1994). *Hairless* controls alternative cell fates in the *Drosophila* epidermis. *Dev.* **120:** 1433-1441.

Scott, M. P. and O'Farrell, P. H. (1986). Spatial programming of gene expression in early *Drosophila embryogenesis. Annu. Rev. Cell Biol.* **2:** 49-80.

Seeger, M., Tear, G., Ferres-Marco, D., and Goodman, C. S. (1993). Mutations affecting growth cone guidance in *Drosophila*: genes necessary for guidance toward or away from the midline. *Neuron.* **10:** 409-426.

Selleck, S. B., Gonzalez, C., Glover, D. M. and White, K. (1992). Regulation of the G1-S transition in postembryonic neuronal precursors by axon ingrowth. *Nature.* **355:** 253-255.

Serafini, T., Kennedy, T. E., Galko, M. J., Mirzayan, C., Jessell, T. M. and Tessier-Lavigne, M. (1994). The netrins define a family of outgrowth-promoting proteins homologous to *C. elegans* UNC-6. *Cell.* **78:** 409-424.

Serikaku, M. A. and O'Tousa, J. E. (1994). *sine oculis* is a homeobox gene required for *Drosophila* visual system development. *Genetics.* **138:** 1137-1150.

Shah, N. M., Marchionni, M. A., Isaacs, I., Stroobant, P., and Anderson, D. J. (1994). Glial growth factor restricts mammalian neural crest stem cells to a glial fate. *Cell.* **77:** 349-360.

Shimizu, C., Akazawa, C., Nakanishi, S., and Kageyama, R. (1995). MATH-2, a mammalian helix-loop-helix factor structurally related to the product of *Drosophila* proneural gene *atonal*, is specifically expressed in the nervous system. *Eur. J. Biochem.* **229:** 239-248.

Silver, J., Lorenz, S. E., Wahlstein, D., and Coughlin, J. (1982). Axonal guidance during development of the great cerebral commissures: descriptive studies, *in vivo*, on the role of preformed glial pathways. *J. Comp. Neurology.* **210:** 10-29.

Silver, J. (1993). Glia-neuron interactions at the midline of the developing mammalian brain and spinal cord. *Perspectives on Developmental Neurobiology.* **1:** 227-236.

Silver, J., Edwards, M. A., and Levitt, P. (1993). Immunocytohistochemical demonstration of early appearing astroglial structures that form boundaries and pathways along axon tracts in the fetal brain. *J. Comp. Neurology.* **328:** 415-436.

Singson, A., Leviten, M. W., Bang, A. G., Hue, X. H., and Posakony, J. W. (1994). Direct downstream targets of proneural activators in the imaginal disc include genes involved in lateral inhibitory signaling. *Genes and Dev.* **8:** 2058-2071.

Sivron, T. and Schwartz, M. (1995). Glial cell types, lineages, and response to injury in rat and fish: implications for regeneration. *Glia.* **13:** 157-165.

Skeath, J. B. and Carroll, S. B. (1991). Regulation of *achaete-scute* gene expression and sensory organ pattern formation in the *Drosophila* wing. *Genes and Dev.* **5:** 984-995.

Skeath, J. B., Panganiban, G., Selegue, J., and Carroll, S. B. (1992). Gene regulation in two dimensions: the proneural *achaete* and *scute* genes are controlled by combinations of axis-patterning genes through a common intergenic control region. *Genes and Dev.* **6:** 2606-2619.

Skeath, J. B., and Carroll, S.B. (1994). The *achaete-scute* complex: generation of cellular pattern and fate within the *Drosophila* nervous system. *FASEB J.* **8:** 714-721.

Skeath, J. B., Panganiban, G., and Carroll, S. B. (1994). The *vnd* gene controls proneural gene expression at two distinct steps during neuroblast formation in *Drosophila. Dev.* **120:** 1517-1524.

Smouse, D., Goodman, C. S., Mahowald, A. P., and Perrimon, N. (1988). *polyhomeotic*: a gene required for the embryonic development of axon pathways in the CNS of *Drosophila. Genes and Dev.* **2:** 830-842.

Snow, D. M., Steindler, D. A., and Silver, J. (1990). Molecular and cellular characterization of the glial roof plate of the spinal cord and optic tectum: a possible role for a proteoglycan in the development of an axon barrier. *Dev. Biol.* **138:** 359-376.

Sonnenfeld, M. J. and Jacobs, J. R. (1995). Apoptosis of the midline glia during *Drosophila* embryogenesis: a correlation with axon contact. *Dev.* **121:** 569-578.

Sperry, R. W. (1963). Chemoaffinity in the orderly growth of nerve fiber patterns and connections *PNAS.* **50:** 703-710.

Steindler, D. A. (1993). Glial boundaries in the developing nervous system. *Annu. Rev. Neurosci.* **16:** 445-470.

Stemple, D. L. and Anderson, D. J. (1992). Isolation of a stem cell for neurons and glia from the mammalian neural crest. *Cell.* **71:** 973-985.

Stoeckli, E. T. and Landmesser, L. T. (1995). Axonin-1, Nr-CAM, and Ng-CAM play different roles in the in vivo guidance of chick commisural neurons. *Neuron.* **14:** 1165-1179.

Stretavan, D. W. (1990). Specific routing of retinal ganglion cell axons at the mammalian optic chiasm during embryonic development. *J. Neurosci.* **10:** 1995-2007.

Stretavan, D. W. and Reichardt, L. F. (1993). Time-lapse video analysis of retinal ganglion cell axon pathfinding at the mammalian optic chiasm: growth cone guidance using intrinsic chiasm cues. *Neuron.* **10:** 761-777.

Stretavan, D. W. (1993). Pathfinding at the mammalian optic chiasm. *Curr. Op. Neurobio.* **3:** 45-52.

Sonnenfeld, M. J. and Jacobs, J. R. (1995). Apoptosis of the midline glia during *Drosophila* embryogenesis: a correlation with axon contact. *Dev.* **121:** 569-578.

Struhl, G., Fitzgerald, K., and Greenwald, I. (1993). Intrinsic activity of the Lin-12 and Notch intracellular domains *in vivo. Cell.* **74:** 331-345.

Swiatek, P. J., Lindsell, C. E., Franco del Amo, F., Weinmaster, G., and Gridley, T. (1994). Notch1 is essential for postimplantation development in mice. *Genes and Dev.* **8:** 707-719.

Technau, G. M. and Campos-Ortega, J. A. (1987). Cell autonomy of expression of neurogenic genes of *Drosophila melanogaster. PNAS.* **84:** 4500-4505.

Tejedor, F., Zhu, X. R., Kaltenbach, E., Ackerman, A., Baumann, A., Canal, I., Heisenberg, M., Fischbach, K-F. and Pongs, O. (1995). *minibrain*: a new protein kinase family involved in postembryonic neurogenesis in *Drosophila. Neuron.* **14:** 287-301.

Temple, S. and Raff, M. C. (1986). Clonal analysis of oligodendrocyte development in culture: evidence for a developmental clock that counts cell divisions. *Cell.* **44:** 773-779.

Tessier-Lavigne, M., Placzek, M., Lumsden, A. G. S., Dodd, J., and Jessell, T. M. (1988). Chemotrophic guidance of developing axons in the mammalian central nervous system. *Nature.* **336:** 775-778.

Tessier-Lavigne. (1995). Eph receptor tyrosine kinases, axon repulsion, and the development of topographic maps. *Cell.* **82:** 345-348.

Thomas, J. B., Bastiani, M. B., Bate, M., and Goodman, C. S. (1984). From grasshopper to *Drosophil*a: a common plan for neuronal development. *Nature.* **310**: 203-207.

Tix, S., Minden, J. S. and Technau, G. M. (1989). Pre-existing neuronal pathways in the developing optic lobes of *Drosophila. Dev.* **105:** 739-746.

Tolbert, L. P. and Oland, L. A. (1989). A role for glia in the development of organized neuropilar structures. *TINS*. **12:** 70-75.

Tolbert, L. P. and Oland, L. A. (1990). Glial cells form boundaries for developing insect olfactory glomeruli. *Exp. Neurology*. **109:** 19-28.

Tomlinson, A. and Ready, D. F. (1987). Neuronal differentiation in the *Drosophila* ommatidium. *Dev. Biol.* **120:** 366-376.

Torrence, S. A., Law, M. I., and Stuart, D. K. (1989). Leech neurogenesis. *Dev. Biol.* **136:** 40-60.

Truman, J. W. and Bate, M. (1988). Spatial and temporal patterns of neurogenesis in the central nervous system of *Drosophila melanogaster*. *Dev. Biol.* **125:** 145-157.

Turner, D. L. and Cepko, C. L. (1987). A common progenitor for neurons and glia persists late in rat retina development. *Nature.* **328:** 131-136.

Turner, D. L., Snyder, E. Y., and Cepko, C. L. (1990). Lineage independent determination of cell type in the embryonic mouse retina. *Neuron*. **4:** 833-845.

Udolph, G., Prokop, A., Bossing, T., and Technau, G. M. (1993). A common precursor for glia and neurons in the embryonic CNS of *Drosophila* gives rise to segment-specific lineage variants. *Dev.* **118:** 765-775.

Uemura, T., Shepherd, S., Ackerman, L., Jan, L. Y., and Jan, Y-N. (1989). *numb*, a gene required in determination of cell fate during sensory organ formation in *Drosophila* embryos. *Cell.* **76:** 477-491.

Valverde, F. and Lopez-Mascaraque, L. (1991). Neuroglial arrangements in the olfactory glomeruli of the hedgehog. *J. Comp. Neur.* **307:** 658-674.

Van Vactor, D., Sink, H., Fambrough, D., Tsoo, R., and Goodman, C. S. 91993). Genes that control neuromuscular specificity in *Drosophila*. *Cell.* **73:** 1137-1153.

Vassin, H., Bremer, K. A., Knust, E., and Campos-Ortega, J. A. (1987). The neurogenic gene *Delta* of *Drosophila melanogaster* is expressed in neurogenic territories and encodes a putative transmembrane protein with EGF like repeats. *EMBO J.* **6:** 3431-3440.

Vervoort, M., Zink, D., Pujol, N., Victoir, K., Dumont, N., Ghysen, A., and Dambly-Chaudiere, C. (1995). Genetic determinants of sense organ identity in *Drosophila*: regulatory interactions between *cut* and *poxn*. *Dev.* **121:** 3111-3120.

Vescovi, A. L., Reynolds, B. A., Fraser, D. D., and Weiss, S. (1993). bFGF regulates the proliferative fate of unipotent (neuronal) and bipotent (neuronal/astroglial) EGF-generated CNS progenitors. *Neuron.* **11:** 951-966.

Walter, J., Henke-Fahle, S., and Bonhoeffer, F. (1987). Avoidance of posterior tectal membranes by temporal retinal axons. *Dev.* **101:** 909-913.

Weinmaster, G., Roberts, V. J., and Lemke, G. (1991). A homologue of *Drosophila Notch* expressed during mammalian development. *Dev.* **113:** 199-205.

Weinmaster, G., Roberts, V. J., Lemke, G. (1992). A second mammalian *Notch* gene. *Dev.* **116:** 931-941.

Wetts, R. and Fraser, S. E. 1988. Multipotent precursors can give rise to all major cell types of the frog retina. *Science.* **239:**1142-1145.

Wharton, K. A., Johansen, K. M., Xu, T., and Artavanis-Tsakonis, S. (1985). Nucleotide sequence from the neurogenic locus *Notch* implies a gene product that shares homology with proteins containing EGF like repeats. *Cell.* **43:** 567-581.

White, K. and Kankel, D. R. (1978). Patterns of cell division and cell movement in the formation of the imaginal nervous system in *Drosophila melanogaster*. *Dev. Biol.* **65:** 296-321.

Wizenmann, A., Thanos, S., Boxberg, Y. V. and Bonhoeffer, F. (1993). Differential reaction of crossing and non-crossing rat retinal axons on cell membrane preparations from the chiasm midline: an *in vitro* study. *Dev.* **117:** 725-735.

Wolff, T. and Ready, D. F. (1991). The beginning of pattern formation in the *Drosophila* compound eye: the morphogenetic furrow and the second mitotic wave. *Dev.* **113:** 841-850.

Woolsey, T. A. (1990). Peripheral alteration and somatosensory development. In *Development of Sensory Systems in Mammals*. (ed. J. R. Coleman). pp. 461-516. John Wiley and Sons: New York.

Xiong, W-C. and Montell, C. (1995). Defective glia induce neuronal apoptosis in the *repo* visual system of *Drosophila*. *Neuron*. **14:** 581-590.

Yang, X., Yeo, S., Dick, T. and Chia, W. (1993). The role of a *Drosophila* POU homeodomain gene in the specification of neural precursor cell identity in the developing embryonic CNS. *Genes and Dev.* **7:** 504-516.

Young, J. Z. (1991). The concept of neuroglia. In *Glial-Neuronal Interactions*. (ed. N. J. Abbott). pp. 1-18. New York: New York Academy of Sciences.

Younossi-Hartenstein, A., Tepass, U., and Hartenstein, V. (1993). Embryonic origin of the imaginal disc of the head of *Drosophila melanogaster*. *Roux's Arch. Dev. Biol.* **203:** 60-73.

Zackenfels, K., Oppenheim, R. W., Rohrer, H. (1995). Evidence for an important role of IGF-1 and IGF-2 for the early development of chick sympathetic neurons. *Neuron*. **14:** 731-741.

Zhang, Y., Ungar, A., Fresquez, C., and Holmgren, R. (1994). Ectopic expression of either the *Drosophila gooseberry-distal* or *gooseberry-proximal* gene causes alterations of cell fate in the epidermis and central nervous system. *Dev.* **120:** 1151-1161.

Zipursky, S. L. and Rubin, G. M. (1994). Determination of neuronal cell fate; lessons from the R7 neuron of *Drosophila*. *Annu. Rev. Neurosci.* **17:** 373-397.

"He who hath clean hands and a pure heart is O.K. in my book,
but he that fools around with barnyard animals has gotta be watched."
from Woody Allen's *Love and Death*, via Kankel, et. al., 1980

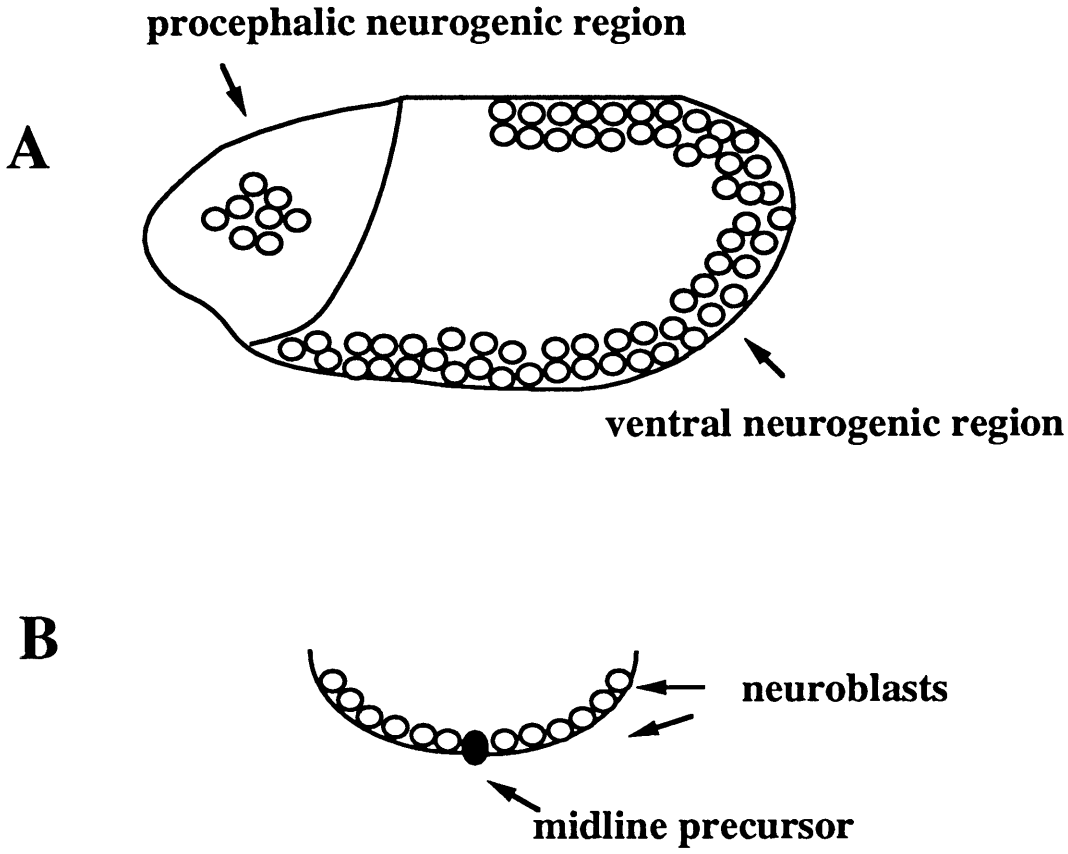
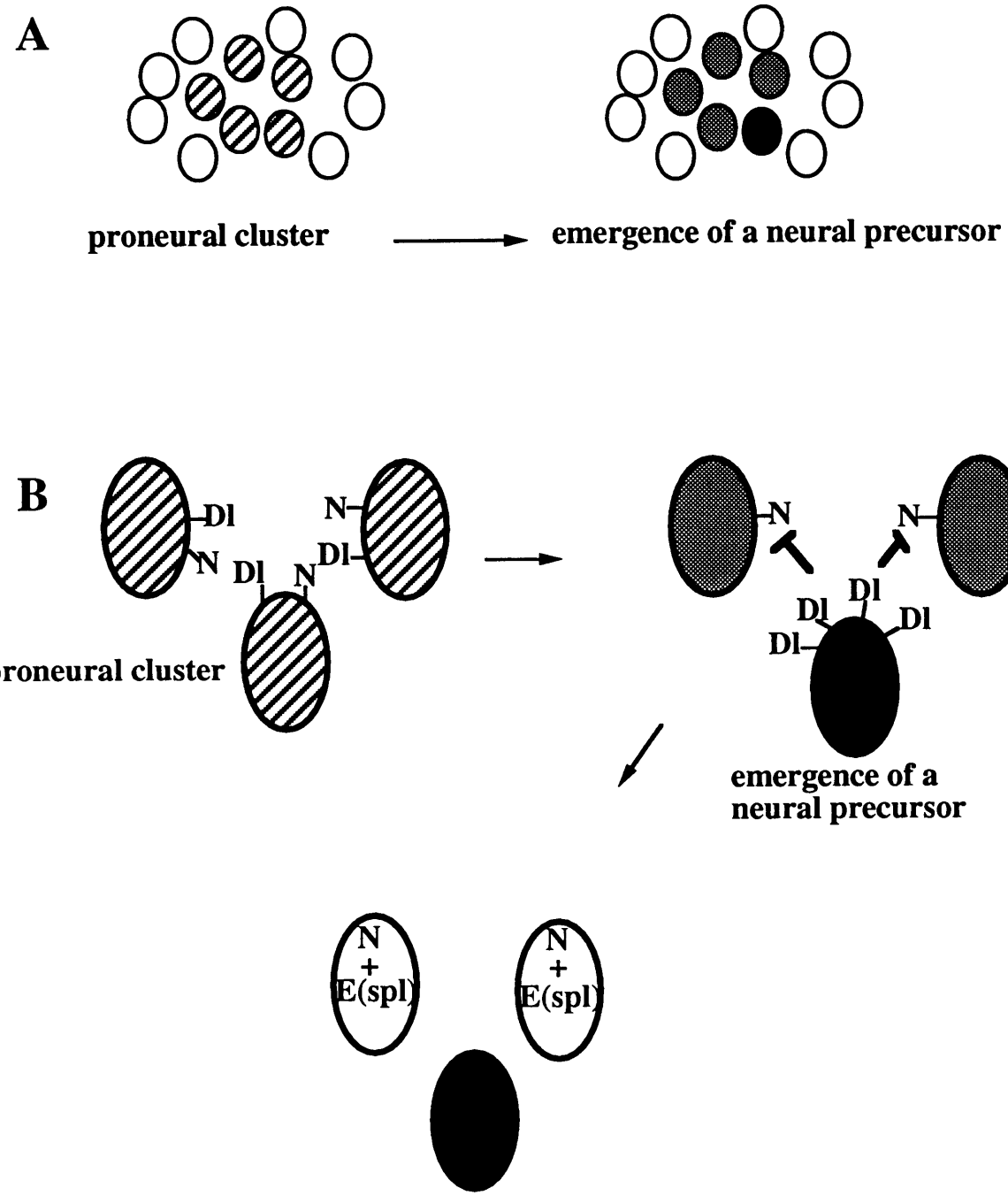

A
procephalic neurogenic region
ventral neurogenic region
B
neuroblasts
midline precursor
C
asymmetric
neuroblast divisions
neuroblast
ganglion mother
cell
neuron or
glial cell

Figure 2: Schematic diagrams detailing lateral specification.

(A) All cells within a proneural cluster initially show equivalent levels of proneural gene expression (stippled circles). Following lateral specification (see below), the neural precursor upregulates proneural gene expression (filled circle), while the remaining cells within the proneural cluster down regulate proneural gene expression (grey circles). (B) All cells within a proneural cluster (stippled circles) express equivalent amounts of the neurogenic loci [eg. *Notch* (*N*) and *Delta* (*Dl*)]. One cell within a proneural cluster will, stochastically, express slightly higher levels of the *N* ligand, *Dl*. This cell (filled circle) upregulates its own expression of the proneural genes. Neighboring cells receive the *Dl* signal via the *N* receptor and downregulate proneural gene expression (grey circles). Signal transduction downstream of *N* induces the expression of the *enhancer of split* [*E(spl)*] transcripts, and cells adopt an epidermal fate (open circles). Figures are modified from Jan and Jan, 1994; Knust, 1994.



differentiation of neural and non-neural cells

Figure 3: Schematic representation of the early development of the vertebrate nervous system.

(A) Cross sections through two germ layers are shown. Signals from the mesoderm (filled rectangle) are received by the ectoderm (open rectangle). (B) The neural plate is induced within the ectoderm (stippled rectangle). (C) The neural plate folds to form the neural tube (stippled structure), which generates all structures within the central nervous system. A population of cells, the neural crest, migrates from the dorsal aspect of the neural tube (solid circles) to generate the peripheral nervous system. The mesodermally derived notochord is found ventral to the neural tube.

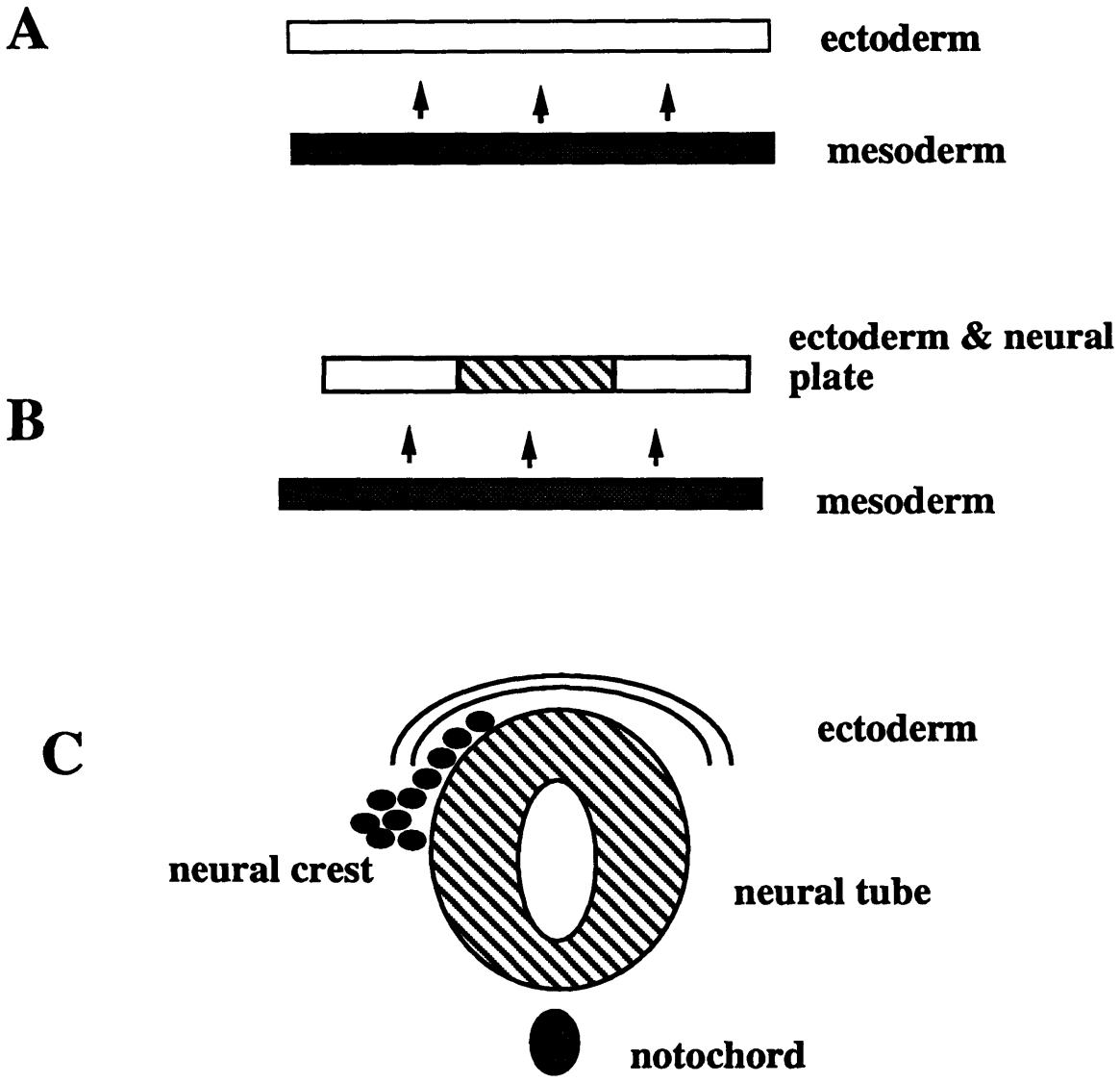
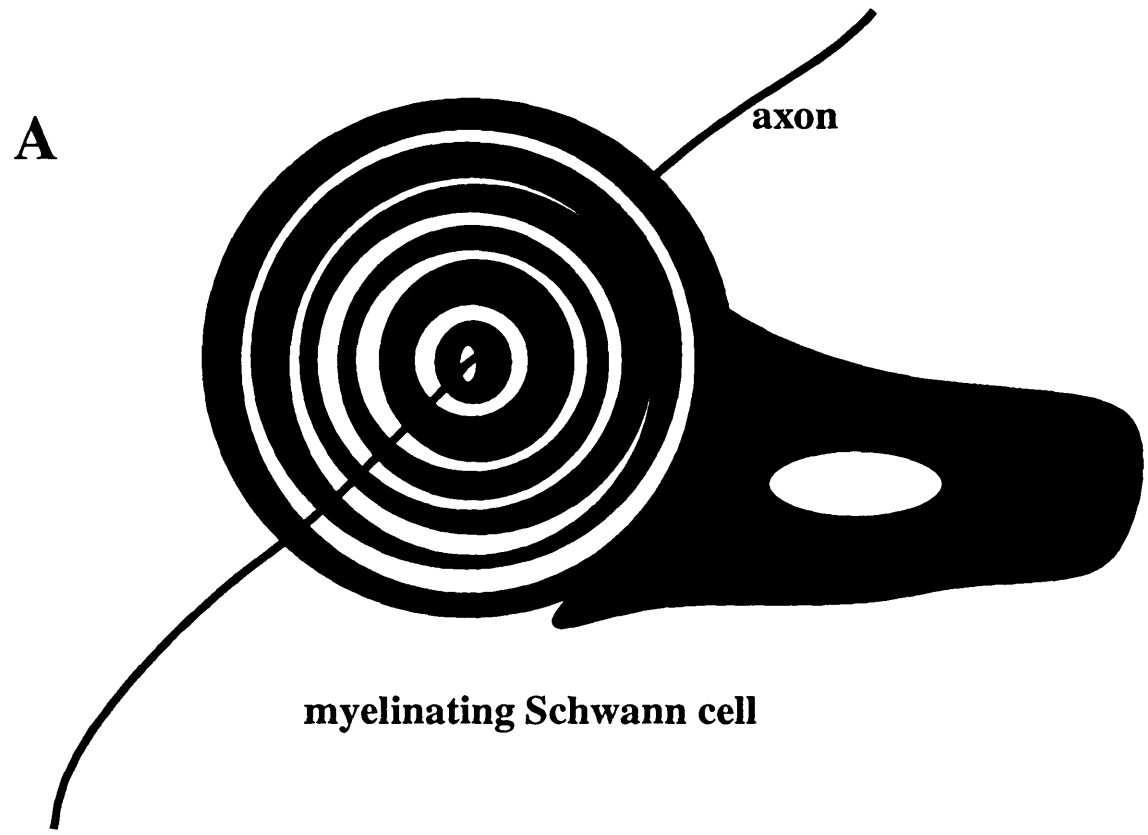

A
ectoderm
mesoderm
B
ectoderm & neural plate
mesoderm
C
ectoderm
neural crest
neural tube
notochord

Figure 4: Two classes of Schwann cells within the vertebrate peripheral nervous system.

(A) Myelinating Schwann cells ensheath single axons in multiple layers of myelin. (B) Non-myelinating Schwann cells extend cytoplasmic processes between multiple axons within single nerves.


A
axon
myelinating Schwann cell

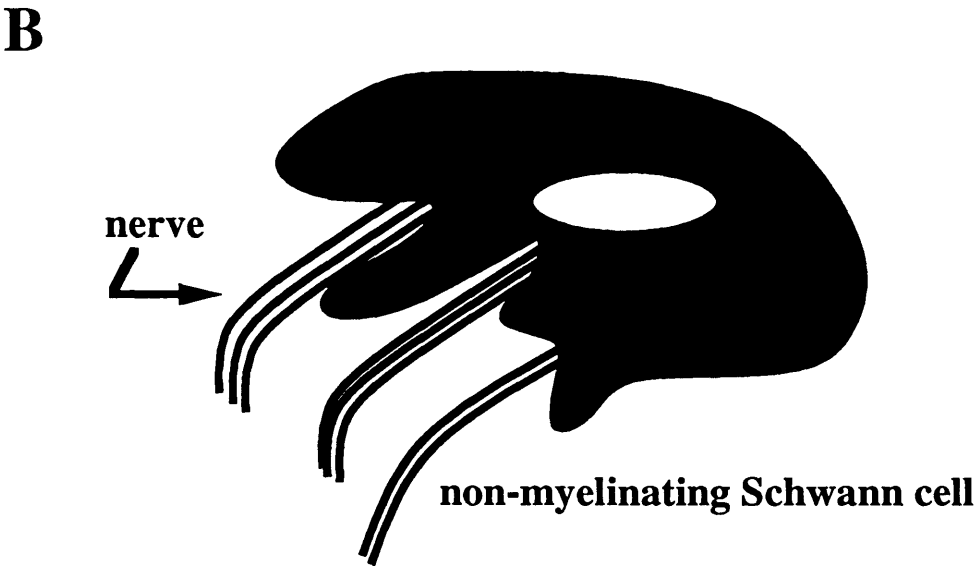
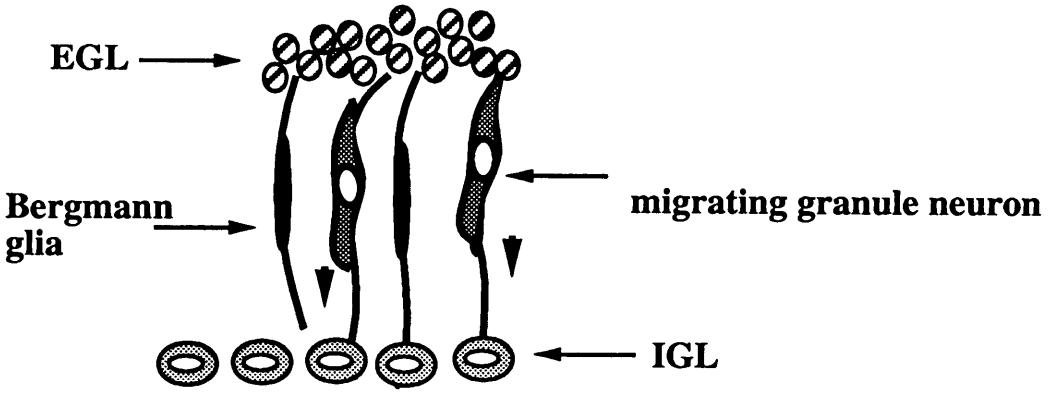

B
nerve
non-myelinating Schwann cell

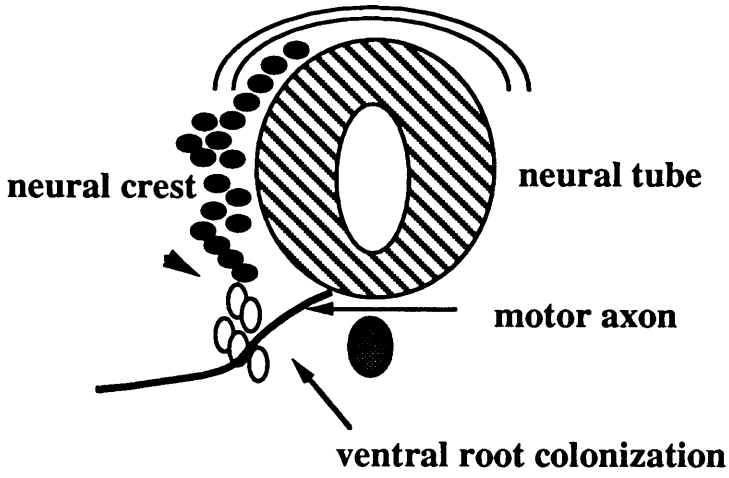
Figure 5: Schematic diagrams of neural and glial migration.

In all panels, the direction of migration is indicated with arrowheads. (A) In the vertebrate central nervous system, cerebellar granule neurons are generated in the external germinal layer (EGL; stippled circles). Post-mitotic neurons migrate from the EGL to their final positions in the internal granule layer (IGL) along glial cell processes. These specialized glia are called Bergmann glia. Figure modified from Hatten and Heinz, 1995). (B) Within the vertebrate peripheral nervous system, a subset of the neural crest (solid circles) colonizes the ventral root (open circles). Motor axons are required to stop migrating crest cells at the appropriate site within the ventral root. (C) At the *Drosophila* midline, commissural axons are initially adjacent to one another. The posterior migration of midline glial cells is required to separate the commissures. Figure modified from Klämbt et. al., 1991.

A


EGL
Bergmann glia
migrating granule neuron
IGL

B


neural crest
neural tube
motor axon
ventral root colonization

C

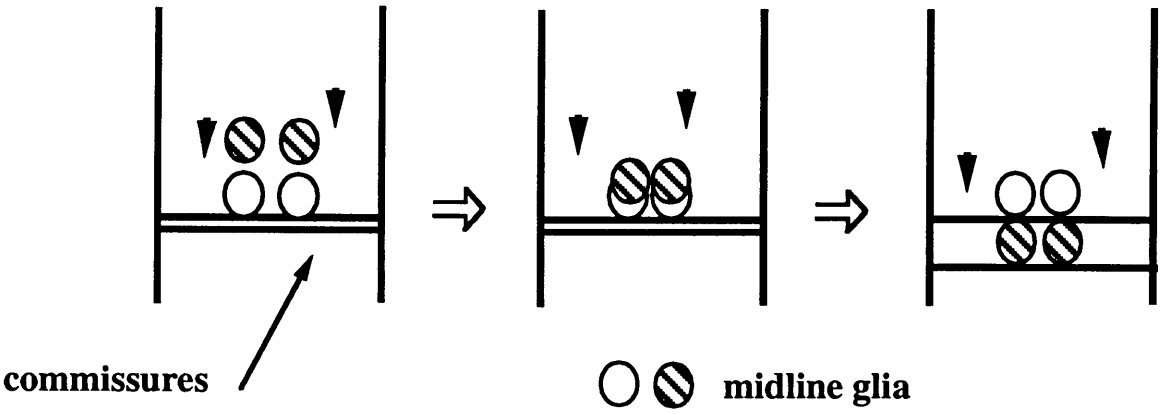
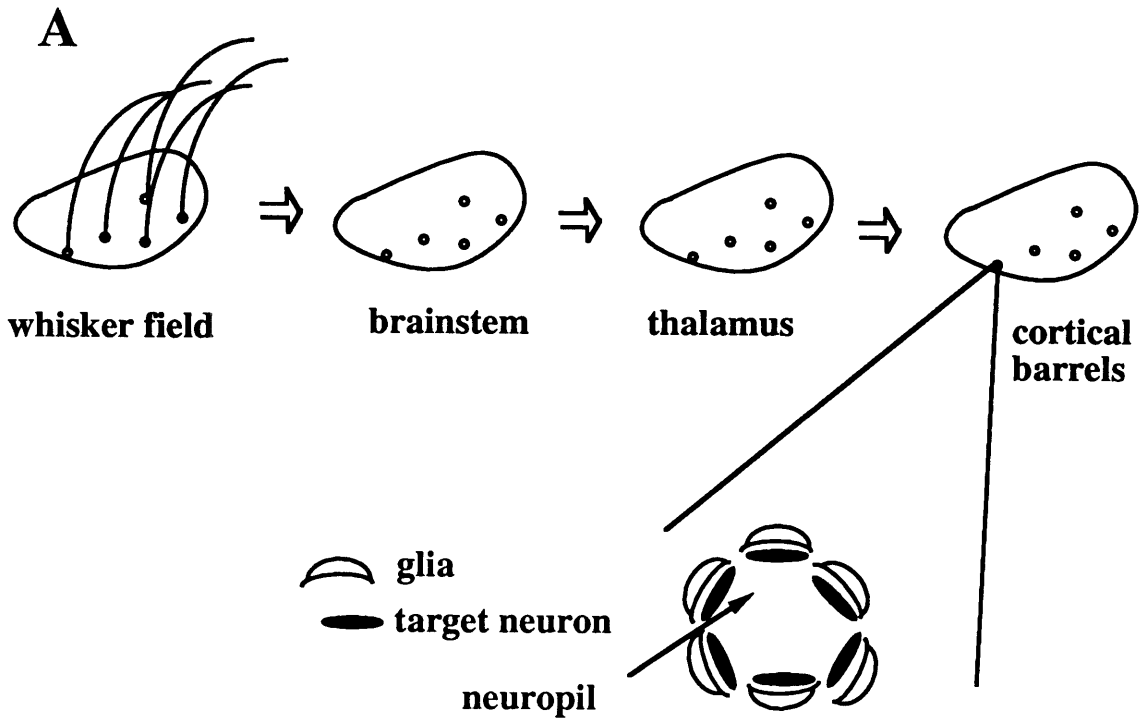

commissures
midline glia

Figure 6: Schematic representations of glial cell boundaries.

(A) Diagram of cortical barrels. In rodents, sensory axons from the face transmit information through the brainstem and thalamus into the cortex. The pattern of facial whiskers is maintained in all three areas of the brain. Within the cortex, afferent input is segregated into "barrels", which consist of glial cells and target neurons. Synapses between thalamic and cortical axons are formed within the barrels. Figure is modified from Woolsey, 1990. (B) Diagram of glomeruli in the *Manduca sexta* antennal lobe. Antennal sensory axons transmit information into the antennal lobe. In response to sensory axon innervation, glia surrounding the antennal lobe migrate into glomeruli within the neuropil. Sensory and antennal lobe axons synapse within the glomeruli. Figure is modified from Tolbert and Oland, 1990.


A
whisker field
brainstem
thalamus
cortical barrels
glia
target neuron
neuropil

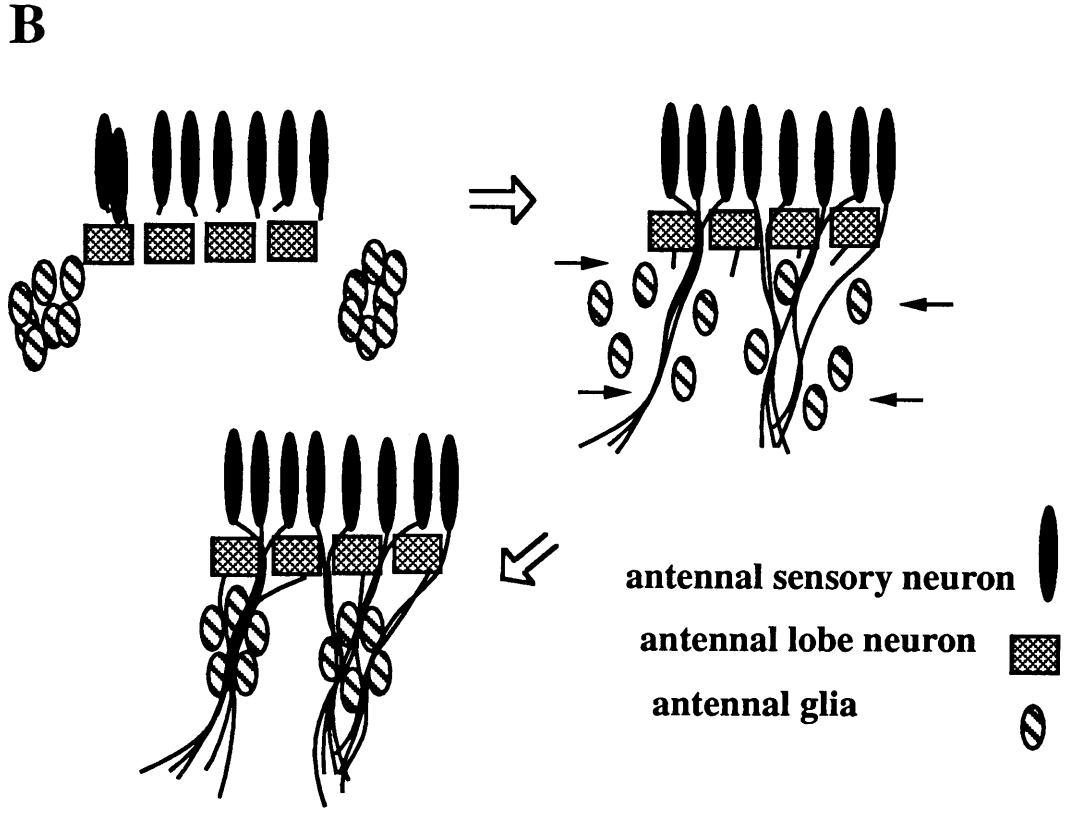

B
antennal sensory neuron
antennal lobe neuron
antennal glia

Figure 7: Schematic diagrams of axonal projections.
(A) Cells within the vertebrate eye project retinotopically to the optic tectum. Information received within the nasal region (solid circles) of the eye is transmitted to the posterior portion of the tectum. Information received within the temporal region (stippled circles) of the eye is transmitted to the anterior portion of the tectum. Figure is modified from Garrity and Zipursky, 1995. (B) Glial cells prefigure the axon tracts along the *Drosophila* midline. The longitudinal tracts are shown as solid lines parallel to the anterior/posterior axis. The commissural tracts are shown as solid lines perpendicular to the anterior/posterior axis. The intersegmental nerves are shown as solid lines extending laterally from the longitudinal tracts. The longitudinal, midline, and intersegmental boundary cell glia are indicated. Figure is modified from Jacobs and Goodman, 1989. (C) Retinal ganglion cell axons project through the optic chiasm en route to the brain. Cells located in the ventral-temporal region of the retina (filled circles) project axons ipsilaterally, while most ganglion cells (grey circles) project contralaterally. Figure is modified from Stretavan, 1993. (D) Thalamic axons (filled circles) from the lateral geniculate nucles (LGN) innervate the visual cortex (open circles), relying on subplate neurons (stippled circles) for positional cues. Figure is modified from Allendoerfer and Schatz, 1994.

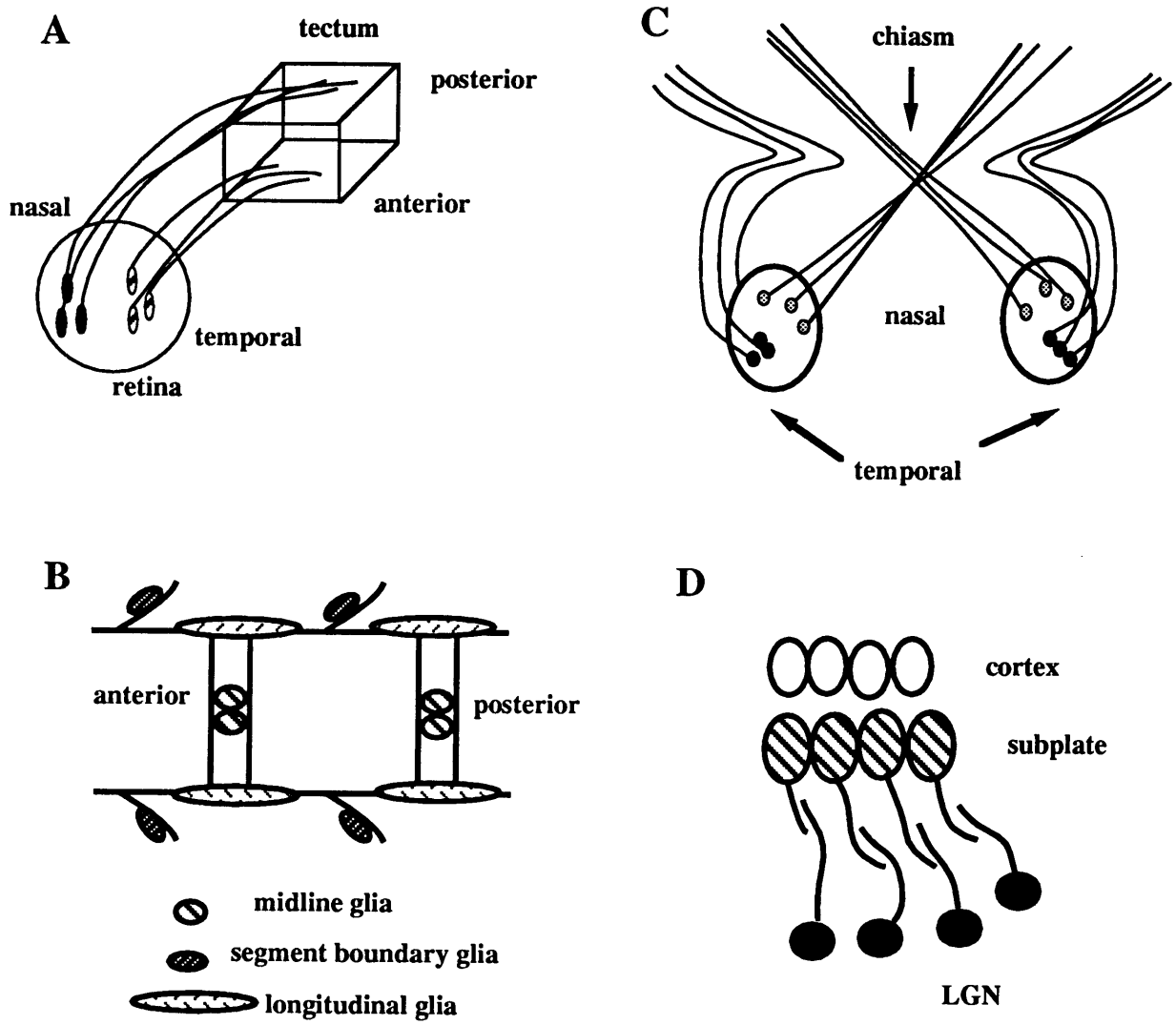
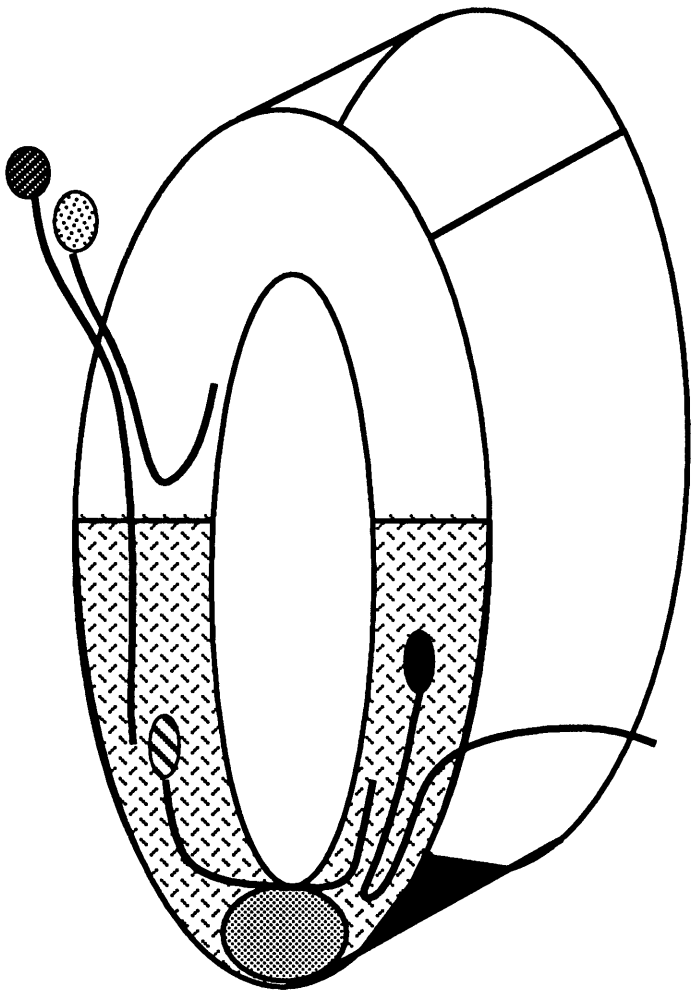

A
tectum
posterior
nasal
anterior
temporal
retina
C
chiasm
nasal
temporal
B
anterior
posterior
midline glia
segment boundary glia
longitudinal glia
D
cortex
subplate
LGN

Figure 8: Schematic diagram of the vertebrate spinal cord.

The spinal cord is shown in cross section. Dorsal is to the top of the page, and the rostral/caudal axis runs perpendicular to the page. Domains of Semaphorin III and netrin I expression are shown. Netrin I attracts commissural axons and repells motor axons. Semaphorin III repulses the axons of NGF responsive neurons, but does not affect NT3 responsive neurons. Figure is modified from Colamarino and Tessier-Lavigne, 1995).



NGF responsive neurons

NT3 responsive neurons

motor neuron

commissural neuron

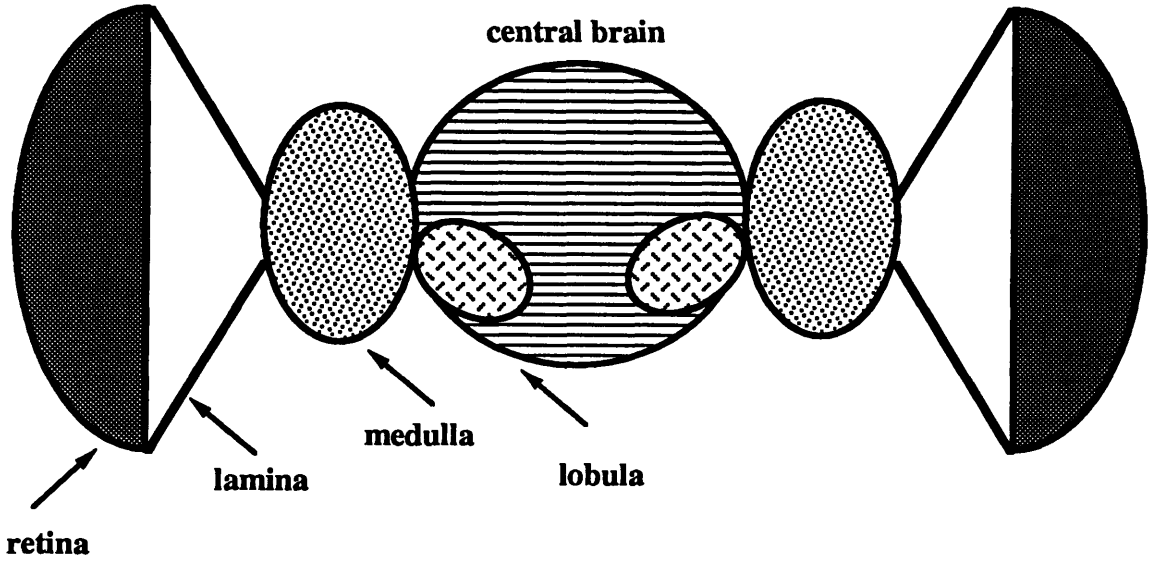
Sema-III

netrin-1

Figure 9: Schematic representations of the *Drosophila* visual system

(A) A cross section through an adult head is shown. Anterior is to the top of the page. The visual system consists of the compound eyes, and bilateral optic ganglia. Moving from the lateral edge of the brain, the ganglia are called the lamina, the medulla and the lobula. (B) A cross section through an ommatidium is shown. Outer photoreceptors (R1-6), drawn as open circles, project into the lamina. Inner photoreceptors (R7,8), drawn as grey circles, project into the medulla. Cone cells (filled triangles) and a subset of pigment cells (stippled ovals) are also shown. Figure is modified from Lawrence and Green, 1979.

A


central brain
medulla
lamina
lobula
retina

B

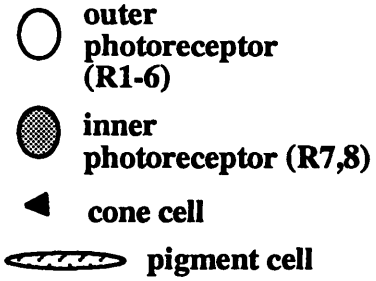
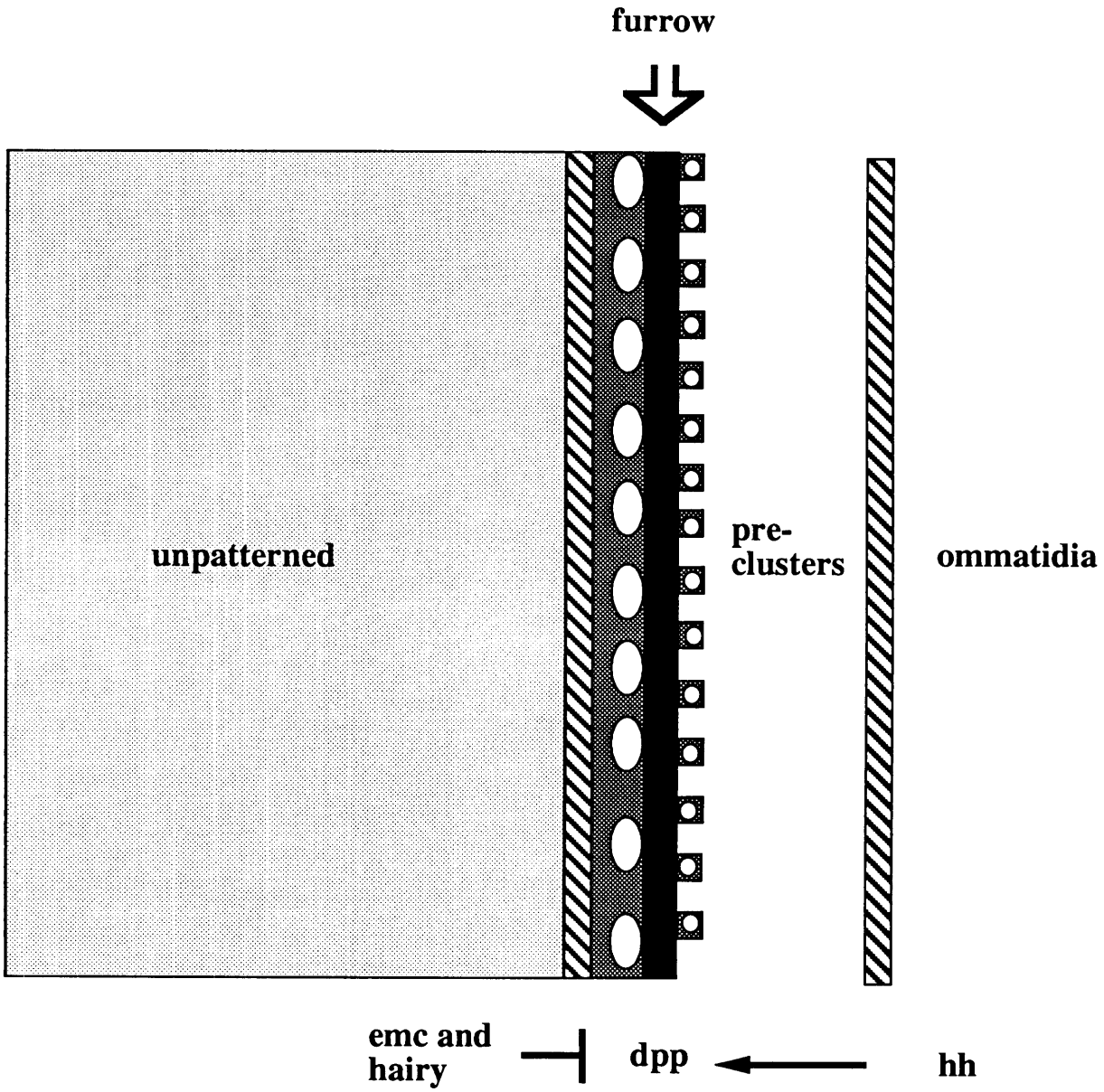

outer photoreceptor (R1-6)
inner photoreceptor (R7,8)
cone cell
pigment cell

Figure 10: Diagram of the developing *Drosophila* retina.

In this representation of the eye disc, anterior is to the left. The unpatterned, anterior portion of the eye disc is shaded (light grey). Two mitotic zones are shown as stippled rectangles, and the locations of ommatidia and pre-clusters are indicated.

Movement of the furrow requires expression of *dpp* within the furrow. The photoreceptors produce *hh*, which positively regulates *dpp*. Cells anterior to the furrow express *emc* and *hairy*, negatively regulating *dpp* and restricting it to the furrow.

Differentiation of photoreceptors requires expression of the proneural gene, *atonal* (*ato*) (dark grey rectangles). Anterior to the furrow, *ato* is uniformly expressed along the dorsal/ventral axis. Three neurogenic loci are expressed in the developing eye: *Notch* (*N*), *Delta* (*Dl*) and *scabrous* (*sca*) (white circles). *scabrous* (*sca*) is expressed in clusters of cells anterior to the furrow. *Notch* and *Delta* act within the furrow to restrict both *ato* and *sca* expression to the ommatidial founder cell, R8.


furrow
unpatterned
pre-
clusters
ommatidia
emc and
hairy
dpp
hh
mitosis
atonal and scabrous
Notch, Delta

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

Margaret L. Winberg, Sharon E. Perez and Hermann Steller

This chapter has been previously published: *Development.* **115,** 903-911. The work was done in collaboration with another graduate student. I identified the 3-109 enhancer trap line as a suitable marker for the lamina glia in third instar larvae and in adult animals. I examined the 3-109 expression pattern in non-innervated brains, carried out the mosaic analysis with the 3-109 marker, and collaborated on the birthdating analysis.

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 a 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 cells 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.

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 18°C or 25°C. 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 *CyO* chromosome in PZ, *CyO/+; D2-3, 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 4°C. 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 4°C 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 37°C 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 mg/ml solution of BrdU in Grace's medium (Gibco) for 30 minutes at 25°C. 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*$^+$, *Δ2-3*]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/+; *Δ2-3*/+. 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 18°C 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 (1μg/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, pers. comm.). 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 have been described in the adult lamina neuropil and cortex (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 the non-neuronal 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 sheath glia, positioned between the marginal glial cells of the lamina and the underlying medulla neuropil, and 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 *Δ2-3* 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; Δ2-3 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 IA. 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 IB, 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 lineages, 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 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 transformation 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, 1988; 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 Fig. 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.

We are grateful to C. Klämbt and C. S. Goodman for providing us with glial-specific enhancer trap lines, including 3-109. We thank D. Smith for sharing technical expertise at confocal microscopy, G. Benson for identifying the VP19 enhancer trap line as a suitable candidate for somatic excision analysis, and S. Benzer and K. White for antibodies. We thank A.R. Campos, S. Kunes, C. Wilson and other colleagues in the Steller lab for critical comments on the manuscript. The confocal microscope facility is supported by a Lucille P. Markey grant to the Department of Brain and Cognitive Sciences. This work was supported in part by a Pew Scholars Award to H.S. M.W. and S.P. received predoctoral fellowships from NSF. H.S. is an Assistant Investigator of the Howard Hughes Medical Institute.

References

Ashburner, M. (1989). *Drosophila.* A Laboratory Handbook. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.

Banerjee, U. and Zipursky, S. L. (1990). The role of cell-cell interaction in the development of the *Drosophila* visual system. *Neuron* **4**, 177-187.

Bronner-Fraser, M. and Fraser, S. E. (1989). Developmental potential of avian trunk neural crest cells *in situ*. *Neuron* **3**, 755-766.

Cagan, R. L. and Ready, D. F. (1989). The emergence of order in the *Drosophila* pupal retina. *Devl Biol.* **136**, 346-362.

Campos, A. R., Fischbach, K-F., and Steller, H. (1992). Survival of photoreceptor neurons in the compound eye of *Drosophila* depends on connections within the optic ganglia. *Development* **114**, 355-366.

Carr, V. M. and Simpson Jr, S. B. (1978). Proliferative and degenerative events in the early development of chick dorsal root ganglion cells. *J. Comp. Neurol.* **182**, 727-740.

Cline, T. W. (1978). Two closely linked mutations in *Drosophila melanogaster* that are lethal to opposite sexes and interact with *daughterless*. *Genetics* **90**, 683-698.

Doe, C. D. and Goodman, C. S. (1985). Early events in insect neurogenesis. I. Development and segmental differences in the pattern of neuronal precursor cells. *Devl Biol.* **111**, 193-205.

Fischbach, K-F. (1983). Neural cell types surviving congenital sensory deprivation in the optic lobes of *Drosophila melanogaster*. *Devl Biol.* **95**, 1-18.

Fischbach, K-F. and Technau, G. (1984). Cell degeneration in the developing optic lobes of the *sine oculis* and *small optic-lobes* mutants of *Drosophila melanogaster*. *Devl Biol.* **104**, 219-239.

Frank, E. and Sanes, J. R. (1991). Lineage of neurons and glia in chick dorsal root ganglia: analysis *in vivo* with a recombinant retrovirus. *Development* **111**, 895-908.

Gratzner, H. G. (1982). Monoclonal antibody to 5-bromo and 5-iododeoxyuridine: a new reagent for detection of DNA replication. *Science* **218**, 474-475.

Hafen, E. and Basler, K. (1991). Specification of cell fate in the developing eye of *Drosophila*. *Development Supp.* **1**, 123-130.

Hall, A. K. and Landis, S. C. (1991). Early commitment of precursor cells from the rat superior cervical ganglion to neuronal or non-neuronal fates. *Neuron* **6**, 741-752.

Hofbauer, A. and Campos-Ortega, J. A. (1990). Proliferation pattern and early differentiation of the optic lobes in *Drosophila melanogaster*. *Roux Arch Devl Biol.* **198**, 264-274.

Jacobs, R. J., Hiromi, Y., Patel, N. H. and Goodman, C. S. (1989). Lineage, migration, and morphogenesis of longitudinal glia in the *Drosophila* CNS as revealed by a molecular lineage marker. *Neuron* **2**, 1625-1631.

Jan, L. Y. and Jan, Y. N. (1982). Antibodies to horseradish peroxidase as specific neuronal markers in *Drosophila* and in grasshopper embryos. *Proc. Natl. Acad. Sci. USA* **79**, 2700-2704.

Kankel, D. R., Ferrus, A., Garen, S. H., Harte, P. J., and Lewis, P. E. (1980). The structure and development of the nervous system. In The Genetics and Biology of Drosophila, Vol. 2D, (ed. M. Ashburner and T. R. F. Wright) London: Academic Press.

Laski, F. A., Rio, D. C. and Rubin, G. M. (1986). Tissue-specificity of *Drosophila* P element transposition is regulated at the level of messenger-RNA splicing. *Cell* **44**, 7-19.

Lindsley, D. L. and Grell, E. H. (1968). Genetic Variations of Drosophila melanogaster. Carnegie Institute of Washington.

Meinertzhagen, I. A. (1973). Development of the compound eye and optic lobe of insects. In Developmental Neurobiology of Arthropods, (ed. D. Young) pp. 51-104. Cambridge: Cambridge University Press.

Meyerowitz, E. M. and Kankel, D. R. (1978). A genetic analysis of visual system development in *Drosophila melanogaster*. *Devl Biol.* **62**, 112-142.

Mlodzik, M. and Hiromi, Y. (1992). The enhancer trap method in Drosophila: its application to neurobiology. In Methods in Neuroscience, Vol 9: Gene Expression in Neural Tissues, (ed. P. M. Conn) pp. 397-414. New York: Academic Press.

Moses, K. and Rubin, G. M. (1991). *glass* encodes a site-specific DNA-binding protein that is regulated in response to positional signals in the developing *Drosophila* eye. *Genes and Dev.* **5**, 583-593.

Nordlander, R. H. and Edwards, J. S. (1969a). Postembryonic brain development in the monarch butterfly *Danaus plexippus plexippus* L. I. Cellular events during brain morphogenesis. *Wilhelm Roux' Arch.* **162**, 197-217.

Nordlander, R. H. and Edwards, J. S. (1969b). Postembryonic brain development in the monarch butterfly *Danaus plexippus plexippus* L. II. The optic lobes. *Wilhelm Roux' Arch.* **163**, 197-220.

O'Kane, C. J. and Gehring, W. J. (1987). Detection *in situ* of genomic regulatory elements in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **84**, 9123-9127.

Oland, L. A., Orr, G. and Tolbert, L. P. (1990). Construction of a protoglomerular template by olfactory axons initiates the formation of olfactory glomeruli in the insect brain. *J. Neurosci.* **10**, 2096-2112.

Oland, L. A. and Tolbert, L. P. (1989). Patterns of glial proliferation during formation of olfactory glomeruli in an insect. *Glia* **2**, 10-24.

Poulson, D. F. (1950). Histogenesis, organogenesis and differentiation in the embryo of *Drosophila melanogaster*. In Biology of Drosophila melanogaster, (ed. M. Demerec) New York: Wiley.

Power, M. E. (1943). The effect of reduction in numbers of ommatidia upon the brain of *Drosophila melanogaster*. *J. Exp. Zool.* **94**, 33-71.

Raff, M. C. (1989). Glial cell diversification in the optic nerve. *Science* **243**, 1450-1455.

Ready, D. F. (1989). A multifaceted approach to neural development. *Trends Neurosci.* **12**, 102-110.

Ready, D. F., Hanson, T. E. and Benzer, S. (1976). Development of the *Drosophila* retina, a neurocrystalline lattice. *Devl Biol.* **53**, 217-240.

Robertson, H. M., Preston, C. R., Phillis, R. W., Johnson-Schlitz, D. M., Denz, W. K., and Engels, W. R. (1988). A stable genomic source of P-element transposase in *Drosophila melanogaster*. *Genetics* **118**, 461-470.

Robinow, S. and White, K. (1991). Characterization and spatial distribution of the ELAV protein during *Drosophila melanogaster* development. *J. Neurobiol.* **22**, 443-461.

Rubin, G. M. (1989). Development of the *Drosophila* retina: inductive events studied at single cell resolution. *Cell* **57**, 519-520.

Saint Marie, R. L. and Carlson, S. D. (1983a). The fine structure of neuroglia in the lamina ganglionaris of the housefly, *Musca domestica* L. *J. Neurocytology* **12**, 213-241.

Saint Marie, R. L. and Carlson, S. D. (1983b). Glial membrane specializations and the compartmentalization of the lamina ganglionaris of the housefly compound eye. *J. Neurocytology* **12**, 243-275.

Selleck, S. B. and Steller, H. (1991). The influence of retinal innervation on neurogenesis in the first optic ganglion of *Drosophila*. *Neuron* **6**, 83-99.

Shaw, S. R. and Meinertzhagen, I. A. (1986). Evolutionary progression at synaptic connections made by identified homologous neurones. *Proc. Natl. Acad. Sci. USA* **83**, 7961-7965.

Silver, J. and Rutishauser, U. (1984). Guidance of optic axons *in vivo* by a preformed adhesive pathway on neuroepithelial endfeet. *Devl Biol.* **106**, 485-499.

Simon, J. A., Sutton, C. A., Lobell, R. B., Glaser, R. B. and Lis, J. T. (1985). Determinants of heat shock-induced chromosome puffing. *Cell* **40**, 805-817.

Stark, W. S. and Carlson, S. D. (1986). Ultrastructure of capitate projections in the optic neuropil of Diptera. *Cell Tissue Res.* **246**, 481-486.

Steindler, D. A., O'Brien, T. F., Laywell, E., Harrington, K., Faissner, A. and Schachner, M. (1990). Boundaries during normal and abnormal brain development: *in vivo* and *in vitro* studies of glia and glycoconjugates. *Exp. Neurol.* **109**, 35-56.

Strausfeld, N. J. (1976). Atlas of an insect brain. New York: Springer-Verlag.

Sved, J. (1986). *eyes absent. Dros. Inf. Serv.* **63**, 169.

Tolbert, L. P. and Oland, L. A. (1989). A role for glia in the development of organized neuropilar structures. *Trends Neurosci.* **12**, 70-75.

Tolbert, L. P. and Oland, L. A. (1990). Glial cells form boundaries for developing insect olfactory glomeruli. *Exp. Neurol.***109**, 19-28.

Tomlinson, A. (1988). Cellular interactions in the developing *Drosophila* eye. *Development* **104**, 183-193.

Trujillo-Cenoz, O. (1965). Some aspects of the structural organization of the intermediate retina of Dipterans. *J. Ultrastruct. Res.* **13**, 1-33.

Trujillo-Cenoz, O. and Melamed, J. (1973). The development of the retina-lamina complex in Muscoid flies. *J. Ultrastruct Res.* **42**, 554-581.

Truman, J. W. and Bate, M. (1988). Spatial and temporal patterns of neurogenesis in the central nervous system of *Drosophila melanogaster*. *Devl Biol.* **125**, 145-157.

Turner, D. L., Snyder, E. Y. and Cepko, C. L. (1990). Lineage-independent determination of cell type in the embryonic mouse retina. *Neuron* **4**, 833-845.

Wetts, R., Serbedzija, G. N. and Fraser, S. E. (1989). Cell lineage analysis reveals multipotent precursors in the ciliary margin of the frog retina. *Devl Biol.* **136**, 254-263.

White, K. and Kankel, D. R. (1978). Patterns of cell division and cell movement in the formation of the imaginal nervous system of *Drosophila melanogaster*. *Devl Biol.* **65**, 296-321.

Zipursky, S. L., Venkatesh, T. R., Teplow, D. B. and Benzer, S. (1984). Neuronal development in the *Drosophila* retina: monoclonal antibodies as molecular probes. *Cell* **36**, 15-26.

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*. Anterior is to the left and lateral is to the top of the page in *d-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.

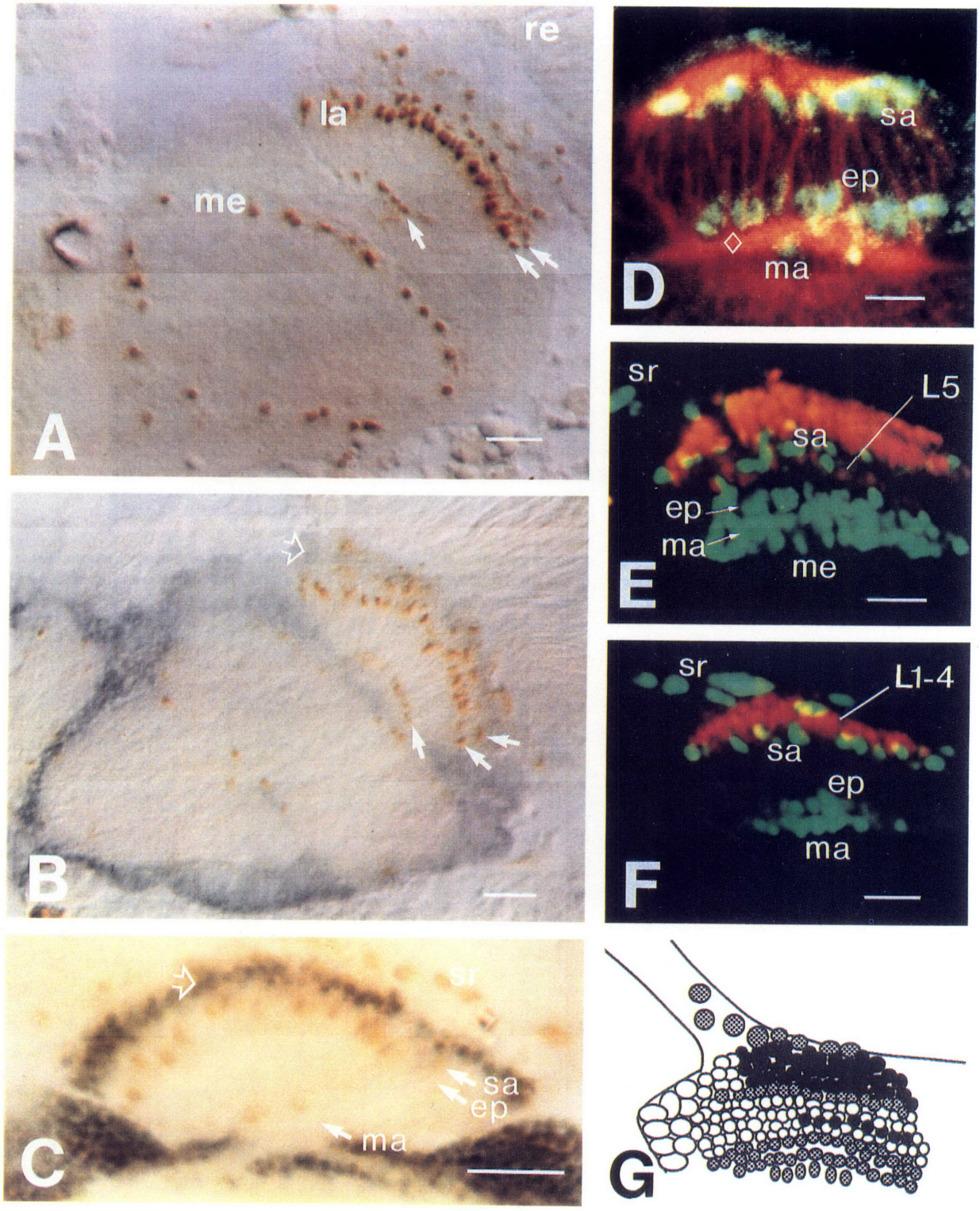

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Fig. 2. Innervation dependent expression of the 3-109 reporter in L-glia.

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.

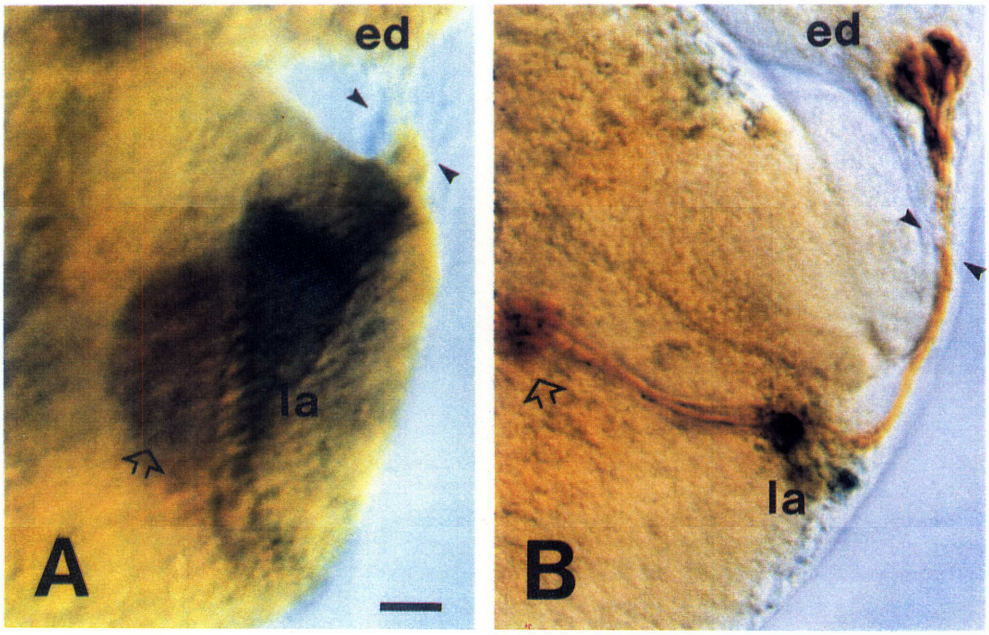
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Fig. 3. Lamina mosaic analysis.

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 *Δ2-3* transposase. An animal which has lost reporter expression in most L-neurons while retaining expression in most L-glia is shown in panel *b*. An animal which has lost reporter expression in most L-glia while retaining expression in most L-neurons is shown 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.

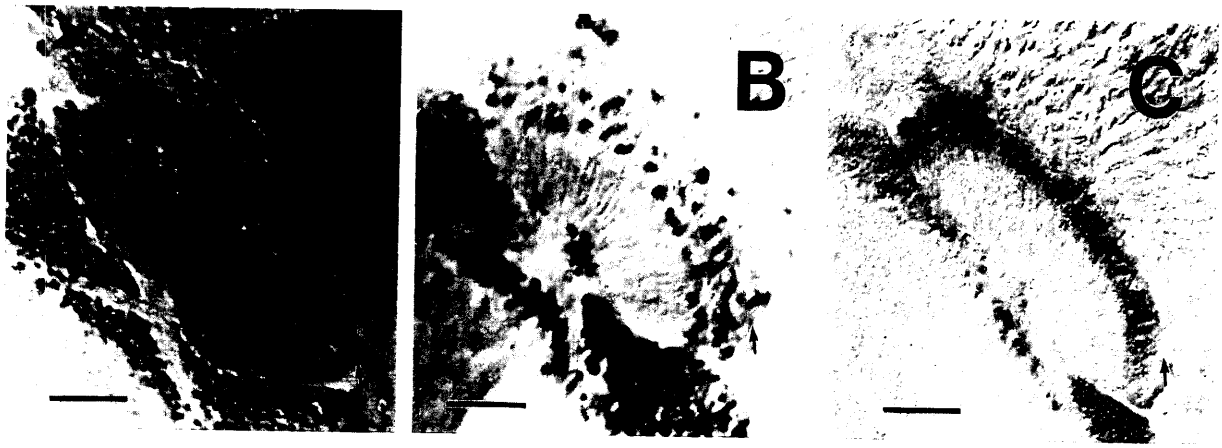
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Fig. 4. BrdU labelling of L-glia in wild type.

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* and *d)* Glial cells. *(b* and *e)* Cells whose precursors incorporated BrdU at the time of the pulse. *(c* and *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.

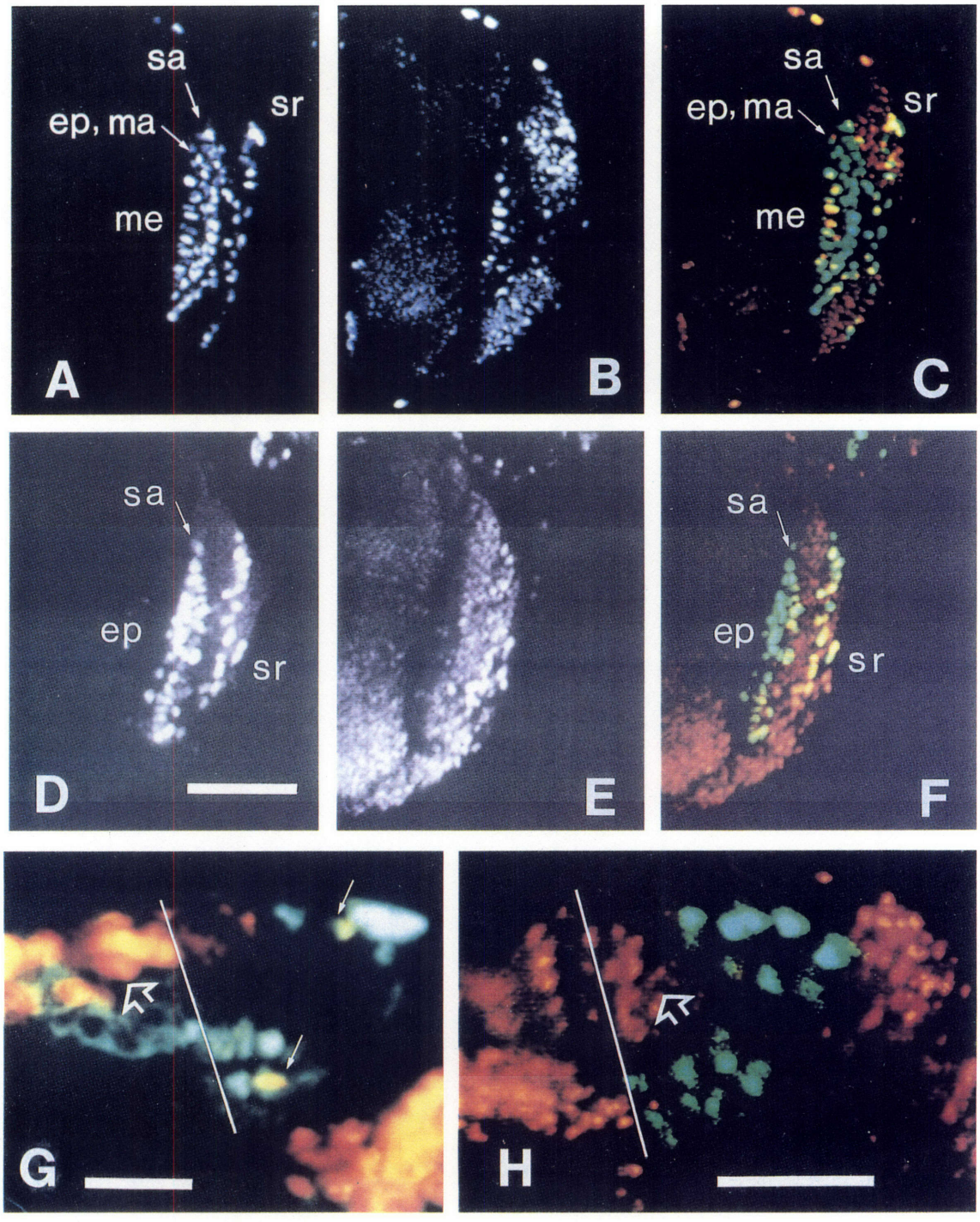
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Fig. 5. Long BrdU pulses in late third instar.

Whole mount, late 3IL brains were stained with anti-β-galactosidase antibody (panel *a*, green/yellow nuclei in panel *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-hr pulse, including extensive incorporation in the proliferation centers (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 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.

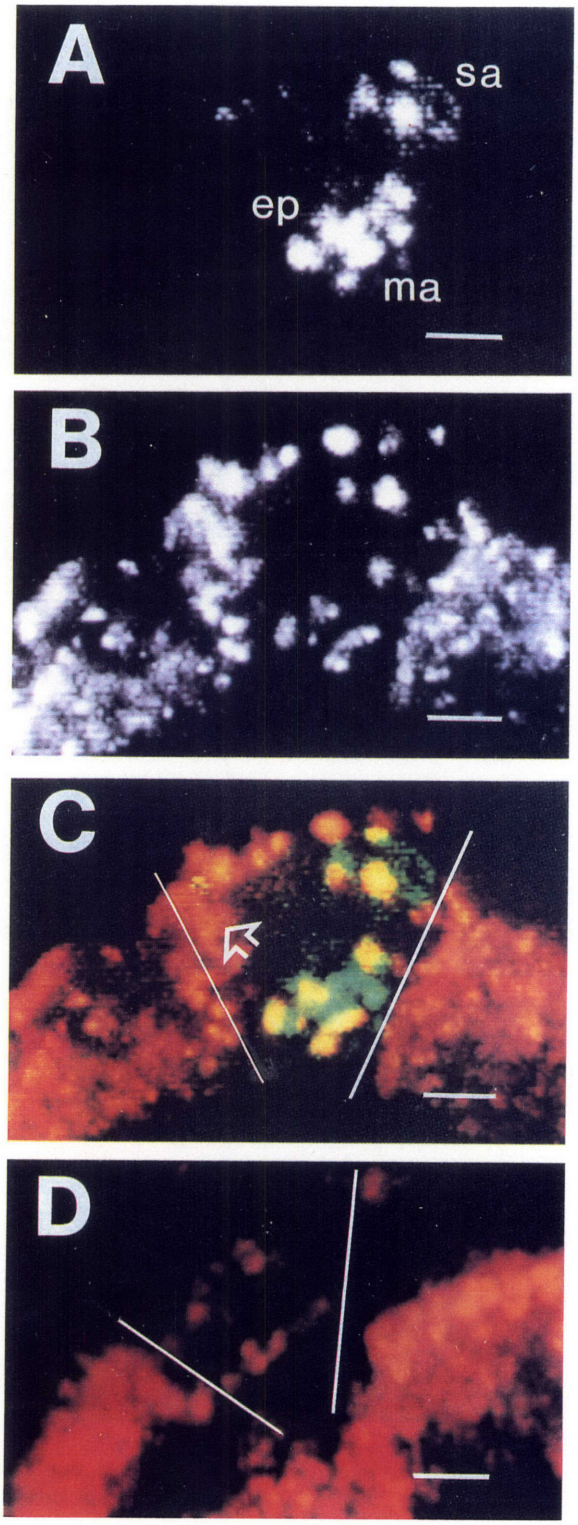
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Table I(A). Somatic mosaic analysis of lamina glia and neurons, using a ubiquitously expressed VP19 reporter as the target for excision.

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.

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		Cells proliferating at time of pulse
	wild-type	mutant	
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 occuring 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

III. Migration of glial cells into the retinal axon target field in *Drosophila melanogaster.*

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This chapter has been accepted for publication, with minor revisions, in the *Journal of Neurobiology*.

SUMMARY

During the development of the *Drosophila* visual system, photoreceptor axons (R-axons) project retinotopically to their targets in the optic lobes. The establishment of this precise pattern of connections does not depend on interactions between adjacent axon bundles, suggesting that R-axons rely on environmental signals for proper pathfinding. Glial cells that are located along the R-axon trajectory are likely candidates to provide guidance cues for R-axon navigation.

This study defines the origin of lamina glia (L-glia), and demonstrates that L-glia migrate into the lamina over a considerable distance. Glia are located in positions at which the R-axons make critical growth choices. In the absence of cues from the eye, several classes of glia migrate to their final positions within the optic lobe anlage and begin to differentiate. Our results are consistent with a role for the L-glia in providing guidance cues to the R-axons.

INTRODUCTION

Neural-glial interactions are important to the development of both vertebrate and invertebrate nervous systems. In some systems, glia provide a substratum for the migration of neurons, or for axon outgrowth. Immature neurons migrate along glia in the vertebrate cerebellar cortex (Rakic, 1972; Hatten, 1990). Axons grow along glial pathways during the formation of the great cerebral commisure in mice (Silver, et.al., 1982), and of the longitudinal connectives in *Drosophila* (Jacobs and Goodman, 1989; Jacobs, 1993). Glia may promote neurite outgrowth (e.g., Wang, et. al., 1994), or provide cues which inhibit growth (Johnston and Gooday, 1991).

Glia appear to pre-pattern both the whisker barrel fields in the mouse somatosensory cortex (reviewed in Steindler, 1993) and the olfactory glomeruli in the *Manduca sexta* antennae (Tolbert and Oland, 1990). Additionally, glia respond to neurons. Glia migrate along axon tracts in the *Drosophila* CNS

(Klämbt, et. al., 1991; Klämbt, 1993), wing disc (Giangrande, 1994), and eye imaginal disc (Choi and Benzer, 1994), as well as in the mammalian optic nerve (Watanabe and Raff, 1988).

In the *Drosophila* visual system, imaginal photoreceptors begin to innervate their target regions during larval life (reviewed in Meinertzhagen and Hanson, 1993). Photoreceptor axons (R-axons) maintain retinal topography as they project into the brain (Kunes et. al., 1993; Kunes and Steller, 1993), and terminate in one of two optic ganglia. The outer photoreceptors (R1-6) project into the first optic ganglion (the lamina) while the inner photoreceptors (R7,8) project deeper, in the medulla. R-axons project retinotopically in the absence of cues from neighboring axons, suggesting that they rely on positional information found along their pathway (Kunes et al, 1993). A recent mutational analysis has indicated that retinal projections may be specifically disrupted at a variety of points along the photoreceptor trajectory, further supporting the existence of local guidance cues (Martin, et. al., 1995).

The synaptic partners of the R-axons would be obvious candidates for providing retinal axon guidance cues. However, in the first optic ganglion, the lamina, R1-6 generate their synaptic partners (Selleck and Steller, 1991), and therefore cannot rely on them for positional cues. Several types of glia are associated with the R-axons, and are found in locations at which the axons change direction or stop growing altogether. To date, these glia have only been described in the fully developed adult animal (Trujillo-Cenoz, 1965; Saint Marie and Carlson, 1983), or in brains that have already received retinal input (Winberg et. al., 1992). The lamina glia and neurons (L-glia, L-neurons) arise from distinct precursor populations (Winberg et. al., 1992), but the location of the glial precursors has not been identified.

In this study, we have investigated whether glia are in the R1-6 target field prior to innervation of the brain. We have used a lineage tracing system (Struhl and Basler, 1993) and an early glial marker, RK2 (Campbell et. al., 1994), to identify the origin of the L-glia. We find that these cells arise from distinct areas of the brain, the dorsal and ventral posterior margins of the developing lamina. We demonstrate that a subset of the optic lobe glia migrates into the lamina anlage prior to, and without, retinal input, and that the L-glia begin to differentiate in the absence of cues from the eye. These data indicate that the

L-glia have the potential to provide retinal axon guidance cues.

METHODS

Stocks

Lines carrying the lineage marker, P[Act5C>Draf+>nuc-lacZ], were provided by G. Struhl (Struhl and Basler, 1993). The FLP recombinase was supplied under the inducible hsp70 promoter (Golic and Lindquist, 1989). An eye specific *sine oculis* (so^1) allele (Fischbach and Technau, 1984; Garren, et. al., 1994) and *disconnected*^{1656} (Heilig et. al, 1991) were used in our analysis. The 3109 enhancer trap line was the gift of C. Klämbt and C. Goodman (see Winberg et. al., 1992). Stocks were maintained on a standard cornmeal medium.

Induction of mosaic clones

The lineage tracing construct, P[Act5C>Draf+>nuc-lac],consists of an actin promoter placed upstream of a *lacZ* gene (Struhl and Basler, 1993). A spacer sequence separates the promoter from *lacZ*, and contains a transcription termination signal. FRT target sites flank the spacer. In animals carrying only the lineage construct, *lacZ* is not expressed. Animals carrying the lineage construct were crossed to those carrying the hsp70-FLP recombinase construct. The resulting progeny were raised at 18° C until the first or second instar, when they were given a mild heat pulse, 32 °C for 25 minutes, to induce the mosaic clones. The animals were returned to 18°C until late third instar, when they were analyzed. At the time of analysis, the larval brain hemispheres had received substantial innervation from the developing eye. Two variations of these conditions were also used. In one experiment, mosaic clones were induced as described above, but the animals were analyzed earlier, during the early third instar. In early third instar larvae, the brain hemispheres have not yet received input from the retina. In a second experiment, the animals were raised at 18°C and given the mild heat pulse later during development, during late second, or early third instar. The animals were subsequently analyzed during late third instar, and the mosaic clones were expected to be smaller than those induced at earlier stages. In all experiments, a small quantity of the recombinase was produced, and, in a few cells, the spacer sequence was excised. In these cells, and in their progeny, *lacZ* was constitutively expressed. Cells within a mosaic patch

were assumed to be more closely related to each other than to cells outside of the clone. Brain hemispheres chosen for analysis had between 1 and 4 well spaced clones.

Immunohistochemistry

Larvae were dissected in 0.1M phosphate buffer (pH 7.2-7.4) and the brain hemispheres were fixed for 30 minutes in 2% paraformaldehyde at room temperature. Antibody staining was carried out as described previously (Winberg et al, 1992). For X-gal/BrdU double labeling, the brains were pulse labeled in vitro with BrdU. The animals were dissected in Grace's media (Gibco BRL) containing BrdU (Boerhinger Mannheim) at a final concentration of 30 ug/ml. Labeling was allowed to continue for 30 minutes at room temperature, and the brains were then fixed for 30 minutes in 2% paraformaldehyde (Polsyciences, Inc.) in 0.1 M Phosphate buffer, pH 7.2-7.4. The brains were rinsed in PBT and stained with X-gal (Bachem). Following X-gal detection, the tissue was processed for BrdU detection by incubating it in 2N HCl in PBT for 20 minutes at room temperature, which exposes the BrdU epitope. For double labeling with anti-ß-galactosidase and BrdU, the tissue was labeled with BrdU and fixed (as above), incubated with the primary antibody to ß-galactosidase and post-fixed with 2% paraformaldehyde prior to treatment with 2N HCl. For staining with Mab44C11 (Bier et. al., 1988), the tissue was treated with collagenase (0.3 units/microliter) (Sigma) for 20 minutes at room temperature prior to fixation, to allow access of the antibody. A 100x collagenase stock (in 50% glycerol, 40 mM $NaPO_4$ buffer, pH 7.4) was diluted in PBS prior to use. The following antibodies were used at a dilution of 1/100-1/200: mouse anti-BrdU (Becton Dickinson), rabbit anti-ß-galactosidase (Cappel), goat anti-mouse-HRP (BioRad), goat anti-rabbit-FITC (Cappel), goat anti-rat-Cy3 and goat anti-mouse-Cy3 (Jackson Immuno), goat anti-HRP-FITC (Cappell; Jan and Jan, 1982) and anti-RK2-5' (Campbell, et. al., 1994). Mab44C11 (Bier et. al., 1988) and Mab24B10 (Zipursky et. al, 1986) were used at 1/5. The anti-RK2-5' antibody was the gift of A. Tomlinson. Mab44C11 was a gift from Y.N. and L. Jan. Mab24B10 was a gift from S. Benzer. Samples were viewed on a Zeiss Axiophot. Immunofluorescence was visualized with a Biorad confocal microscope using software provided by the manufacturer.

RESULTS

Visualization of the retinal axons and lamina glia in the developing brain

By the late third instar larval stage, approximately half of the R-axons present in the adult have innervated the lamina. Lamina development is coincident with retinal input, and the neurons and glia (L-neurons and L-glia) can be distinguished from each other by their characteristic positions in the tissue and with cell type specific markers (reviewed in Meinertzhagen and Hanson, 1993). A third instar brain carrying the 3109 glial enhancer trap line (see Winberg et. al., 1992) is shown in Fig. 1A. It is stained with anti-ß-galactosidase, to detect glia, and co-stained with MAb44C11 (Bier et. al., 1988), to detect the neuronal antigen, elav (Robinow et. al., 1988). The L-glia, shown in red, are found in three distinct layers. The satellite glial layer lies just underneath, and intermixed with, the largest layer of L-neurons, shown in green (L1-4), while the epithelial and marginal glial layers lie deeper in the tissue, beneath a smaller neuronal layer (L5).

In well innervated brains, glia are closely associated with R-axons. Fig. 1B shows a third instar brain, stained with an antibody to the glial antigen, RK2-5' (red) (Campbell, et. al., 1994) and co-stained with anti-HRP (green), which detects all *Drosophila* neurons (Jan and Jan, 1982). Subretinal glia lie alongside the R-axons on the brain's surface. R-axons penetrate the brain and terminate in either the lamina (outer photoreceptors, R1-6) or the medulla (inner photoreceptors, R7,8). The epithelial and marginal L-glial layers flank the R1-6 termini. An additional layer of glia, the medulla glia, are found in two rows, just beneath the marginal L-glial layer.

All cells in the lamina are believed to arise from a large proliferation zone called the outer proliferation center, or, the OPC (White and Kankel, 1978; see Fig. 1C,D). The developing lamina lies posterior to the OPC. New rows of R-axons grow into the lamina along its anterior border. Retinal innervation of the brain is shown schematically in Fig. 1C,D. In lateral view, as diagrammed in Fig. 1C, the lamina appears crescent shaped, and single layers of cells within the tissue are visible in any given focal plane. Conversely, horizontal views of the lamina, as diagrammed in Fig. 1D, show the multiple cell layers within the tissue. The OPC generates a discrete set of L-neuron precursors, designated the lamina neuroblasts (lnb), which are located at the lamina's anterior border (Fig.

1C,D). The lnb surround a depression, or furrow, in the brain's surface. Earlier work had demonstrated that the L-neurons are generated by the lnb (White and Kankel, 1978; Selleck and Steller, 1991), and that the L-glial precursors are distinct from those of the L-neurons (Winberg, et. al., 1992). However the origin of the L-glia, and their migration route into the lamina, have not been described. It has also not been determined whether immature glia are found in the lamina anlage prior to retinal input.

Lineage analysis identifies distinct origin of the L-neurons and L-glia

In order to identify the L-glial precursors, we carried out a mosaic analysis using an FLP based lineage tracing system (Struhl and Basler, 1993). An earlier analysis of mosaic clones in adults indicated that the L-neurons and L-glia arise from different precursor populations (Winberg, et. al., 1992). We analyzed third instar larvae, which enabled us to look at the relationship between cells in the developing lamina and their precursors in the larval proliferation zones. We wanted to determine whether or not there were lineage restrictions within the OPC with respect to the L-glia. One possibility was that many cells within the OPC have the potential to generate L-glia. An alternative possibility was that distinct subset of the OPC generates the L-glia. Mosaic clones were induced in first or second instar larvae. In our analysis of late third instar brain hemispheres (n=454), we found that 10%-30% of mosaic clones included cells in the lamina, and that the lamina clones had two distinct shapes. The first class of clones was radially shaped, while the second class was crescent shaped.

Mosaic clones containing the L-neurons were radial in shape, and ran along the anterior/posterior axis of the brain. The brain shown in Fig. 2A is stained with X-gal, to detect the lineage clone (blue), and co-stained with BrdU (brown), to visualize cell proliferation, thereby indicating the borders of the lamina. Radial patches included cells in the outer proliferation center (OPC), the furrow, and in the body of the lamina (Fig. 2A). Radial clones were examined in brains stained with anti-ß-galactosidase, to detect the lineage patch (green), and co-stained with anti-RK2-5' (Campbell et. al., 1994), to detect glia (red). These clones included L-neurons, but not L-glia (Fig. 2B), indicating that the L-glia do not enter the lamina through the furrow at its anterior edge.

L-glial mosaic clones were crescent shaped, and extended into the lamina from

either its dorsal/posterior or ventral/posterior edge (Fig. 3A-F). In Fig. 3A, a late third instar brain is stained with X-gal, to detect the lineage clone (blue), and co-stained with BrdU (brown), to indicate the borders of the lamina by detecting the proliferation zones. We will refer to these two distinct sites within the OPC as the "glial precursor zones". Glial clones extended into the lamina underneath the neuronal precursor divisions at the dorsal or ventral corners of the lamina (data not shown). Glial clones were examined in brains stained with anti-ß-galactosidase, to detect the lineage patch (green), and co-stained with anti-RK2-5' (Campbell et. al., 1994), to detect glia (red) (Figs. 3B-F and 4). Cells that are within a glial clone, and express the RK2 marker, appear yellow. Crescent shaped clones were viewed horizontally (Fig. 3D), and included all three types of L-glia, but did not include the subretinal or medulla glia.

Mosaic clones containing subretinal glia often included glia in the optic stalk and in the eye disc as well. A clone containing all three types of glia is shown in horizontal view in Fig. 4A. The optic stalk is outlined with white lines, and an arrow indicates the position of subretinal glia on the brain's surface. Earlier work suggested that the retinal basal glia (RBGs) in the eye disc migrate into the disc from the optic stalk along retinal axons (Choi and Benzer, 1994). The subretinal glia appear to be related to at least some of the glia in the optic stalk, and to some of the RBGs (Fig. 4A). However, clones restricted to cells in the optic stalk and the subretinal glia layer have also been observed. These data suggest that, while a multipotent glial precursor, capable of giving rise to all three glia exists, its potency becomes restricted over time. Alternatively, there may be additional glial precursors with fates restricted to either the RBG or subretinal lineages.

One class of medulla glia appear to be related to cells surrounding the ganglion. The medulla is found medial to the lamina. In Fig. 4B, several lineage clones include glia in the medulla, which surround the ganlion's neuropile. The neuropile is indicated with a circle. These clones are also "radial" in shape, but originate deeper in the brain than the radial clones which give rise to L-neurons.

Analysis of L-glial mosaic clones

Induction of glial clones in first or second instar animals gave rise to clones that were quit large by late third instar. However, the clones did not extend much past the lamina's midline (shown in Fig. 3A-C). This observation suggests that

glia in the dorsal lamina arise from the dorsal glial precursor zone, while glia in the ventral lamina arise from the ventral glial precursor zone. It is possible that the lamina consists of dorsal and ventral compartments.

Immature glia migrate into the lamina along its anterior border. We induced mosaic clones in late second instar, or early third instar, animals, and examined the brain hemispheres of late third instar larvae (n=88) (Fig. 3E,F). As expected, we found that these clones were smaller than those which were induced in younger animals (Fig. 3A-D). These small clones often included cells within a single glial layer (data not shown). This supports earlier work which indicated that L-glia within a layer are more closely related to each other than to L-glia in other layers (Winberg, et. al., 1992). The time at which a glial clone was induced correlated with its position along the anterior/posterior axis. Glial clones induced relatively late in the animal's life extended from the glial precursor zones into the anterior lamina, while clones induced in younger animals included glia in the posterior lamina. Our results are consistent with immature glia migrating as far as 35-50 μm, which is the distance between either glial precursor zone and the dorsal/ventral mid-point of the anterior border of a well innervated lamina (see Fig. 3B,C).

Immature glia were often found anterior to the lamina, in positions that anticipated the termination points of R1-6 axons that had not yet grown into the tissue (Fig. 3 B,E,F). In wild type animals, expression of the RK2 glial marker (red) is only seen in cells within the body of the lamina. Glial mosaic clones (green) contained both RK2 positive cells (red), which appeared yellow, and cells which had not yet begun to express the RK2 marker (20%- 50% of the glial clones examined). Immature glia found anterior to the lamina were seen at depths in the tissue that corresponded to the positions of the epithelial and marginal glial layers (see Fig. 1D). The epithelial and marginal layers flank the R1-6 termini, and will be refered to as the "deep L-glia". Glial clones were not observed to extend beyond the lamina by more than 2-3 cell diameters, suggesting that the deep L-glial precursors migrate into the lamina just in advance of retinal input.

L-glia migrate into the lamina prior to retinal innervation

In order for the L-glia to provide positional information to the R-axons, they must be in the lamina anlage prior to the onset of retinal input. We examined whether or not immature glia migrate into the tissue before retinal innervation

begins. We induced mosaic clones in first or second instar larvae, and examined the brain hemispheres (n=52) in early third instar larvae. Retinal innervation begins at about 94 hours after egg laying (AEL). Lineage patches were examined in animals that were between 75-94 hours AEL. In Fig. 5A,B, the mosaic clones are detected via an X-gal stain (blue), and the state of retinal innervation of the brains was assesed by counter-staining with MAb 24B10 (brown) (Zipursky et. al, 1986), an antibody which detects the chaoptin epitope on both larval and imaginal photoreceptor axons. In a "pre-innervation" brain, approximately 70 hours AEL, crescent clones extend into the presumptive lamina from both the dorsal and ventral glial precursor zones (Fig. 5A). Expression of MAb24B10 is only seen in Bolwig's nerve. An innervated brain is shown in Fig. 5B for comparison. In Fig. 5C,D, the mosaic clones are stained with anti-ß-galactosidase (red) and the brains are counter-stained with anti-HRP (green) (Jan and Jan, 1982) to detect axons. In a pre-innervation brain (Fig. 5C), a crescent shaped clone extends into the presumptive lamina from a glial precursor zone. The anti-HRP antibody detects no imaginal photoreceptors, but stains the larval optic nerve. An innervated brain is shown in Fig. 5D for comparison. These data indicate that at least some of the immature L-glia move into the lamina well in advance of axon input from the eye.

L-glia in non-innervated brains

We have demonstrated that the glial precursor zones generate cells which migrate into the lamina prior to retinal innervation. In order to investigate whether migration of immature glia into the lamina requires retinal input at later stages, we compared the distribution of glial cells in wild type brain hemispheres (n=39) to that seen in non-innervated hemispheres (n=66). We examined the glia with an antibody to the RK2 glial marker (Campbell, et. al., 1994). Our non-innervated animals were *sine oculis*^{1} mutants(Fischbach and Technau, 1984; Garren et. al., 1994). This particular allele of *sine oculis* acts in the eye, variably reducing the amount of retinal input to the brain, and does not autonomously affect the development of the lamina. In Figs. 6A-D and 7A-E, the brains are double labelled with the anti-RK2-5' antibody (red) to detect glia, and with anti-HRP (green) (Jan and Jan, 1982), to indicate the position of the R-axons and other

axonal fibers.

In wild type animals, a layer of subretinal glia is found on the brain's surface (Fig. 6A). The subretinal glial layer lies just above the lamina cortex (Fig. 1B,D), and above the most superficial layer of L-glia, the satellite glia (Fig. 6B). Subretinal glial nuclei are larger than satellite glial nuclei. The epithelial and marginal L-glial layers lie deeper in the tissue (one layer shown in Fig. 6C), anterior to the transmedullary fibers. The transmedullary fibers are a useful landmark in non-innervated brains, because they are not affected by retinal input. The medulla neuropile, and one class of medulla glia, are found beneath the marginal L-glia (Fig. 6D).

In non-innervated *sine oculis*^{1} animals, the numbers of the subretinal and the medulla glia (Fig. 7 A,B), as assayed by RK2 expression, are comparable to that in wild type animals (Fig. 6 A,D). This data indicates that the subretinal and marginal glia do not require cues from the retinal axons for their positioning and for the expression of at least one glial marker. In contrast, wild type organization of the L-glia appears to require retinal innervation. Some of the epithelial and marginal glia express the RK2 marker in the absence of retinal input, although they do not appear to express later glial markers (Winberg, et. al., 1992). Fewer cells appear in these deep L-glial layers (Fig. 7 C,D). As in wild type animals, these deep L-glia are located anterior to the transmedullary fibers. In the epithelial layer, glia appear to be clustered around Bolwig's nerve, and additional RK2 positive cells are observed in the glial precursor zones (Fig. 7C). Glia in the marginal layer are arranged in a single row of cells, stretching from from the glial precursor zones into the presumptive lamina (Fig. 7D).

In the absence of retinal input, immature glia appear to accumulate in the precursor zones, and inappropriately express RK2. In non-innervated mutants, the glial precursor zones appear qualitatively larger, as assayed by an independent marker for the L-glial precursors (Perez and Steller, in preparation). These data are consistent with a model in which a few immature glia migrate into the lamina prior to retinal innervation, and begin to differentiate, forming nascent "epithelial" and "marginal" layers. In a wild type animal, these nascent epithelial and marginal layers would flank the termini of the first row of R1-6 axons to grow into the brain.

Retinal innervation of the brain appears to be required in order to trigger the continued migration of immature glia into the lamina. Axon input is also

required for all RK2 expression in the satellite L-glial layer, and for RK2 expression in the majority of the deep L-glia. In a partially innervated *sine oculis*^{1} animals, RK2 expression in the satellite layers is restricted to the glia adjacent to the R-axons (Fig. 7E).

Subretinal glia in the absence of an optic stalk

Earlier work suggested that the subretinal glia migrate through the optic stalk into the brain during third instar life (Cagan and Ready, 1989). In the absence of an optic stalk, no subretinal glia should be observed on the brain's surface. We examined *disconnected* (*disco*) third instar larval brains (n=7). In *disco* mutants, the larval optic nerve does not maintain connections to its targets in the optic lobes (Steller et al, 1987). Most of these mutants display an additional "unconnected" phenotype, in which the optic stalk does not form, and imaginal photoreceptors fail to reach the brain. In Fig. 8A,B, the brains are double labeled with anti-RK2-5', to detect glia (red) and with anti-HRP (green) (Jan and Jan, 1982), to determine the state of retinal innervation of the brain. Fig. 8A shows retinal innervation of the brain and the distribution of the subretinal glia in a wild type hemisphere. Figure 8B shows a similar focal plane of an "unconnected" *disco* hemisphere. In the absence of an optic stalk, RK2 positive cells are found on the surface of the brain in approximately the same location as the subretinal glia are found in wild type hemispheres. However, it appears that there are fewer cells in the subretinal glia position in "unconnected" *disco* brains. Some of the subretinal glia must originate in the brain. The other population, as previously reported (Cagan and Ready, 1989), is likely to derive from the eye disc.

DISCUSSION

We have examined the early development of glial cells in the *Drosophila* visual system. Photoreceptor axons (R-axons) from the developing retina are guided to specific retinotopic positions in the brain. They appear to respond to guidance cues in their local environment (Kunes, et. al, 1993; Martin, et. al, 1995). We have investigated the possibility that the optic lobe glia provide positional information to ingrowing R-axons. The major class of R-axons, R1-6, triggers the proliferation of its target cells, the lamina neurons (L-neurons), and cannot, therefore, rely on them for positional cues (Selleck and Steller, 1991). An analagous situation is found in the vertebrate cerebral cortex. Thalamic axons innervate their target field, before their

synaptic partners have migrated into place. To choose the correct target field, these axons rely on the subplate neurons located beneath the visual cortex (McConnell, et. al, 1989; Ghosh, et. al., 1990).

In the *Drosophila* optic lobe, several classes of glial cells are located along the R-axon trajectory. The glia have only been described once the R-axons have innervated the brain (Trujillo-Cenoz, 1965; Saint Marie and Carlson, 1983; Winberg et. al., 1992). In order to study the lamina glial (L-glia) precursors, we have used a lineage tracing system (Struhl and Basler, 1993) to carry out a mosaic analysis in third instar larvae. We identify the origin of the L-glia, and demonstrate that these cells migrate into the lamina from distinct regions of the outer proliferation center (OPC). L-glia, but not the subretinal and medulla glia, are generated at either the dorsal/posterior or ventral/posterior corners of the lamina, in regions designated the "glial precursor zones". Therefore, some of the glia migrate into the lamina over a considerable distance.

In order for optic lobe glia to provide guidance cues to the R-axons, they must be in position within the tissue before retinal innervation. The distribution and early differentiation of both the subretinal and medulla glia appear normal in the absence of axon input. We present three lines of evidence indicating that at least a subset of the L-glia also migrate into the lamina prior to input from the eye. First, immature L-glia are often found in positions that anticipate the future termination points of R1-6 axons, anterior to the innervated portion of the lamina. Second, we have examined the distribution of immature L-glia in young, "pre-innervation" animals, and have found that their migration into the lamina anlage may precede axon input by as many as twenty hours. Finally, we have examined L-glia in the brains of non-innervated mutants, using a glial specific antibody, anti-RK2 (Campbell et. al, 1994). In wild type animals, the lamina is well innervated, and each row of R1-6 axons terminates between two layers of L-glia, the epithelial and marginal layers. In age matched, non-innervated animals, most of the immature glia remain in the glial precursor zones. However, in these animals, small epithelial and marginal layers are always observed.

Our data are consistent with a model in which immature L-glia migrate into the lamina prior to retinal innervation, and form nascent epithelial and marginal layers. These layers are in the correct position to signal to the first row of R-axons to grow into the brain, perhaps triggering the termination of R1-6. Innervation of the brain is required for the continued migration of glia into the lamina. Each new row of

epithelial and marginal glia could potentially signal to an ingrowing row of R-axons. Such a model would enable the L-glia to serve as guidepost cells, as well as allow the appropriate number of glia to be incorporated into the lamina, such that the L-glial layers are matched in size with both the lamina cortex and the overlying retina.

Previously, it had been shown that the L-glia require retinal innervation for the expression of some glial markers (Winberg, et. al., 1992). We find that expression of an early marker, RK2, in the satellite L-glial layer also appears to require axon input. In contrast, some of the cells within the epithelial and marginal glial layers express RK2 without retinal innervation. This data indicates that the classes of L-glia begin to acquire their distinct fates in the absence of cues from ingrowing R-axons.

There are several examples of neural-glial interactions in *Drosophila*. Glia in the wing disc and optic stalk migrate along axons to their final positions (Choi and Benzer, 1994; Giangrande, 1994). Glia prefigure axon tracts in the CNS (Jacobs and Goodman, 1989; Jacobs, 1993), and aberrant glial development will disrupt CNS structure (Klämbt, 1993).

Drosophila photoreceptor axons are capable of independent navigation (Kunes et. al., 1993), and can properly innervate the optic lobes without guidance cues from other axon fascicles. It has been suggested that the retinal axons rely on cues provided by cells along their trajectory and in their target field. The subretinal glia are located adjacent to the retinal axons as they spread across the brain's surface. Two classes of L-glia, the epithelial and marginal cells, flank the R1-6 termini. Our data indicate that these glia move into their appropriate positions in the optic lobe in the absence of retinal innervation. We suggest that the subretinal glia, and some of the L-glia, which migrate into the lamina over a considerable distance, may play instructive roles during axon ingrowth.

Acknowledgments: We are grateful to A. Tomlinson and G. Struhl, for providing us with the RK2 antibody and the lineage tracing line. We thank D. Smith, for expert assistance with confocal microscopy. We thank S. Kunes, P. McCaw, K. Roberg, and members of the Steller lab for critical comments on the manuscript. The confocal microscope facility is supported by a Lucille P. Markey grant to the

Dept. of Brain and Cognitive Sciences. S. P. received a pre-doctoral fellowship from NSF. H. S. is an Associate Investigator of the Howard Hughes Medical Institute.

REFERENCES

Bier, E., Ackerman, L., Barbel, S., Jan, L.Y., and Jan, Y.N. (1988). Identification and characterization of a neuron specific nuclear antigen in *Drosophila*. *Science*. **240:** 913-916.

Cagan, R.L. and Ready, D.F. (1989). The emergence of order in the *Drosophila* pupal retina. *Devl. Biol.* **136:** 346-362.

Campbell, G., Göring, H., Lin, T., Spana, E., Andersson, S., Doe, C.Q., and Tomlinson, A. (1994). RK2, a glial specific homeodomain protein required for embryonic nerve cord condensation and viability in *Drosophila*. *Development*. **120:** 2957-2966.

Cheyette, B.N.R., Green, P.J., Martin, K., Garren, H., Hartenstein, V. and Zipursky, S.L. (1994). The *Drosophila sine oculis* locus encodes a homeodomain containing protein required for the development of the entire visual system. *Neuron*. **12:** 977-996.

Choi, K. and Benzer, S. (1994). Migration of glia along photoreceptor axons in the developing *Drosophila* eye. *Neuron*. **12:** 423-431.

Feltri, M.L., Scherer, S.S., Nemni, R., Kamholz, J., Vogelbacker, H., Scott, M.O., Canal, N., Quaranta, V., and Wrabetz, L. (1994). ß4 integrin expression in myelinating Schwann cells is polarized, developmentally regulated and axonally dependent. *Development*. **120:** 1287-1301.

Fischbach, K.-F., and Technau, G. (1984). Cell degeneration in the developing optic lobes of the *sine oculis* and *small optic lobes* mutants of *Drosophila melanogaster*. *Devl. Biol.* **104:** 219-239.
19.

Giangrande, A. (1994). Glia in the fly wing are clonally related to epithelial cells and use the nerve as a pathway for migration. *Development*. **120:** 523-534.

Ghosh, A., Antonini, A., McConnell, S.K., and Shatz, C.J. (1990). Requirement for subplate neurons in the formation of thalamocortical connections. *Nature*. **347:**179-181.

Golic, K.S. and S., Lindquist. (1989). The FLP recombinase of yeast catalyzes site specific recombination in the *Drosophila* genome. *Cell*. **5:** 499-509.

Hatten, M.E. (1990). Riding the glial monorail: a common mechanism for glial guided neuronal migration in different regions of the developing mammalian brain. *Trends in Neuroscience.* **13(5):** 179-184.

Heilig, J.S., Freeman, M., Laverty, T., Lee, K.J., Campos, A.R., Rubin, G.M., Steller, H. (1991). Isolation and characterization of the *disconnected* gene of *Drosophila melanogaster. Embo J.* **10(4):** 809-815.

Jacobs, J.R. (1993). Perturbed glial scaffold formation precedes axon tract malformation in *Drosophila* mutants. *J. Neurobiol.* **24(5):** 611-626.

Jacobs, J.R. and Goodman, C.S. (1989). Embryonic development of axon pathways in the *Drosophila* CNS. I. A glial scaffold appears before the first growth cones. *J. Neuroscience.* **9(7):** 2402-2411.

Jan, L.Y. and Jan, Y.N. (1982). Antibodies to horseradish peroxidase as specific neuronal markers in *Drosophila* and in grasshopper embryos. *Proc. Natl. Acad. Sci. USA.* **79:** 2700-2704.

Johnston, A.R. and Gooday, D.J. (1991). *Xenopus* temporal retina neurites collapse on contact with glial cells from caudal tectum in vitro. *Development.* **113:** 409-417.

Klämbt, C. (1993). The *Drosophila* gene *pointed* encodes two ets like proteins which are involved in the development of the midline glia. *Development.* **117:** 163-176.

Klämbt, C., Jacobs, R.J., and Goodman, C.S. (1991). The midline of the *Drosophila* central nervous system: a model for the genetic analysis of cell fate, cell migration, and growth cone guidance. *Cell.* **64:** 801-815.

Kunes, S. and Steller, H. (1993). Topography in the *Drosophila* visual system. *Curr. Opin. Neurobiol.* **3(1):** 53-59.

Kunes, S., Wilson, C., and Steller, H. (1993). Independent guidance of retinal axons in the developing visual system of *Drosophila. J. Neuroscience.* **13(2):** 752-767.

Martin, K.A., Poeck, B., Roth, H., Ebens, A.J., Ballard, L.C., and Zipursky, S.L. (1995). Mutations disrupting neuronal connectivity in the *Drosophila* visual system. *Neuron.* **14:** 229-240.

McConnell, S. K., Ghosh, A., and Shatz, C.J. (1989). Subplate neurons pioneer the first axon pathway from the cerebral cortex. *Science.* **245:** 978-982.

Meinertzhagen, I.A. and Hanson, T.E. (1993). The development of the optic lobe. In *The Development of Drosophila melanogaster,* (eds. M. Bate and A. Martinez-Arias). pp. 1363-1491. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Rakic, P. (1972). Mode of cell migration in the superficial layers of the fetal monkey neocortex. *J. Comp. Neurobiol.* **145:** 61-84.

Robinow, S., Campos, A.R., Yao, K.M., White, K. (1988). The *elav* gene product of *Drosophila*, required in neurons, has three RNP consensus motifs. *Science.* **242:** 1570-1572.

Saint Marie, R.L. and Carlson, S.D. (1983). The fine structur of neuroglia in the lamina ganglionaris of the housefly, *Musca domestica* L. *J. Neurocytology.* **12:** 213-241.

Selleck, S.B. and Steller, H. (1991). The influence of retinal innervation on neurogenesis in the first optic ganglion of *Drosophila*. *Neuron.* **6:** 83-99.

Silver, J., Lorenz, Suzanne, E., Wahlsten, D., and Coughlin, J. (1982). Axonal guidance during development of the great cerebral commissures: descriptive and experimental studies in vivo, on the role of preformed glial pathways. *J. Comp. Neurol.* **210:** 10-29.

Spreyer, P., Kuhn, G., Hanemann, C.O., Gillen, C., Schaal, H., Kuhn, R., Lemke, G., and Müller, H.W. (1991). Axon-regulated expression of a schwan cell transcript that is homologous to a "growth arrest-specific" gene. *EMBO J.* **12(12):** 3661-3668.

Steller, H., Fischbach, K.-F., and Rubin, G.M. (1987). *disconnected*: a locus required for neuronal pathway formation in the visual system of *Drosophila*. *Cell.* **50:** 1139-1153.

Steindler, D.A. (1993). Glial boundaries in the developing nervous system. In *Annual Review of Neuroscience,* (ed) (location:press) **16:** 445-470.

Struhl, G. and Basler, K. (1993). Organizing activity of wingless protein in *Drosophila*. *Cell.* **115:** 903-911.

Tolbert, L.P., and Oland, L.A. (1989). A role for glia in the development of organized neuropilar structures. *TINS.* **12:** 70-75.

Trujillo-Cenoz, O. (1965). Some aspects of the structural organization of the intermediate retina of dipterans. *J. Ultrastructure Research.* **13:** 1-33.

Wang, L.C., Baird, D.H., Hatten, M.E., and Mason, C.A. (1994). Astroglial differentiation is required for support of neurite outgrowth. *J. Neuroscience.* **14(5):**3195-3207.

Watanabe, T. and Raff, M.C. (1988). Retinal astrocytes are immigrants from the optic nerve. *Nature*. **332(6167):** 834-837.

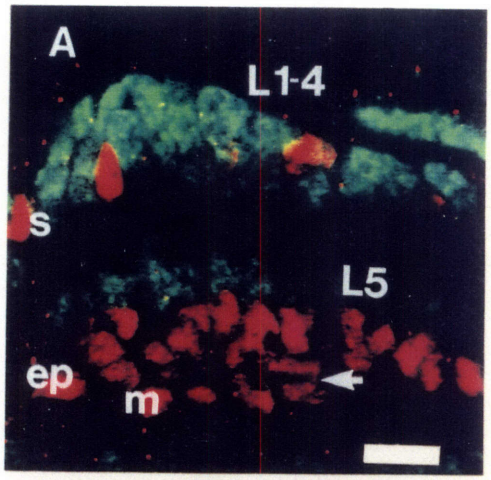
White, K. and Kankel, D.R. (1978). Patterns of cell division and cell movement in the formation of the imaginal nervous system of *Drosophila melanogaster*. *Devl. Biol.* **104:** 219-239.

Winberg, M.L., Perez, S.E., and Steller, H. (1992). Generation and early differentiation of glial cells in the first optic ganglion of *Drosphila melanogaster*. *Development*. **115:** 903-911.

Zipursky, S., Venkatesh, T., and Benzer, S. (1986). From monoclonal antibody to gene for a neuron-specific glycoprotein in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **82**: 1855-1859.

Fig. 1. Retinal axons are associated with glial cells in the third instar lamina.

Anterior is to the left in all panels (A) Lamina neurons and glia occupy distinct positions within the lamina, and are shown in horizontal view. The brain is taken from a third instar larva carrying an enhancer trap line, 3109, which marks glia. Glial nuclei are visualized with anti-ß-galactosidase (red) and neuronal nuclei are visualized with MAb44C11 (green). An arrow indicates the position of the R1-6 termini. The two layers of lamina neurons (L1-4 and L5) and three layers of lamina glia (satellite layer, s; epithelial layer, ep; marginal layer, m) are visible. An arrow indicates the position of the R1-6 termini. (B) There is a correlation between glial cell position and the alteration of the R-axon trajectory. A third instar brain is shown in horizontal view, stained with anti-HRP (green) to detect axons and co-stained with anti-RK2-5' (red) to detect glial nuclei. An arrow indicates the position of the R1-6 termini. The subretinal (sr) and medulla (me) glial layers are indicated. (C) Schematic of a third instar brain and eye disc complex in lateral view. The eye disc (ed) and lamina (la) are indicated. A single R-axon and subsets of the outer proliferation center (OPC) and lamina neuroblasts (lnb) are shown. Cells in S phase are indicated by stippled circles. (D) Schematic of a horizontal view of the third instar lamina (la) and R-axons. Within the lamina, the subretinal glia (sr) are represented by rectangles; lamina neurons by filled circles (L1-4, L5); lamina glia by squares (satellite layer, s; epithelial layer, e; marginal layer, m); medulla glia (me) by ovals. The outer proliferation center is indicated (OPC), as are the lamina neuroblasts (lnb). Cells in S phase are indicated by stippled ovals. Scale bars: A, 20 μm; B, 16μm.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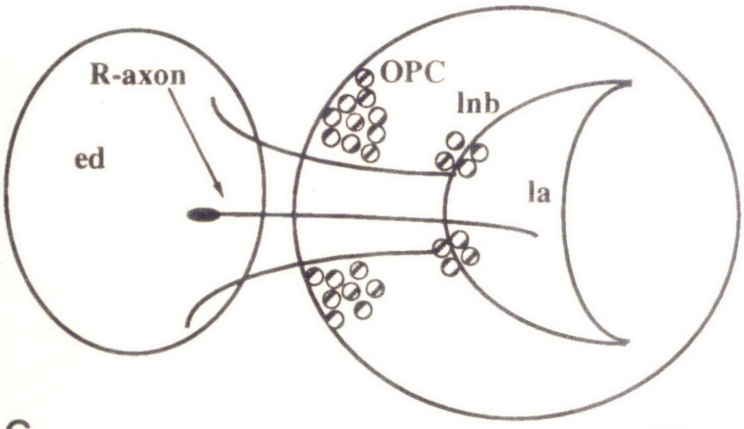
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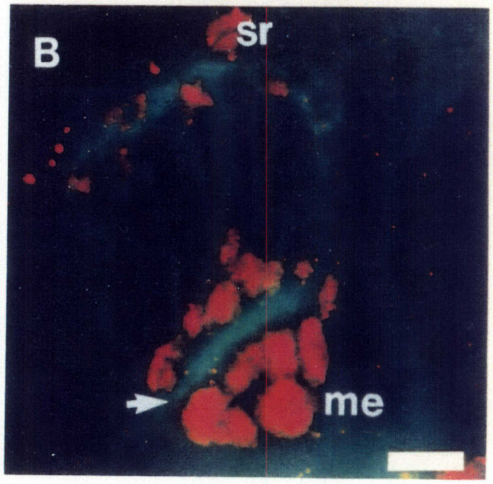
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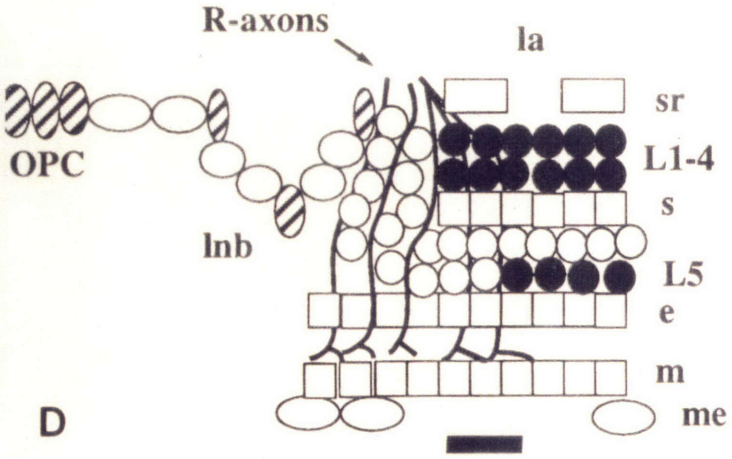
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Fig. 2. Analysis of L-neuron mosaic clones.

All clones were induced in first or second instar larvae. Anterior is to the left in both panels. (A) Lateral view of a lineage clone. The brain is pulse labelled with BrdU (brown) and co-stained with X-gal (blue) to detect the lineage clone. The lamina (la) and the OPC are indicated. An open arrow marks the position of lamina neurogenesis. (B) A horizontal view of a lineage clone. The brain is stained with anti-ß-galactosidase (green) to detect the clone and co-stained with anti-RK2-5' (red) to detect glia. An open arrow marks the position of lamina neurogenesis. Scale bars: A, 12.5 μm; B, 20 μm.

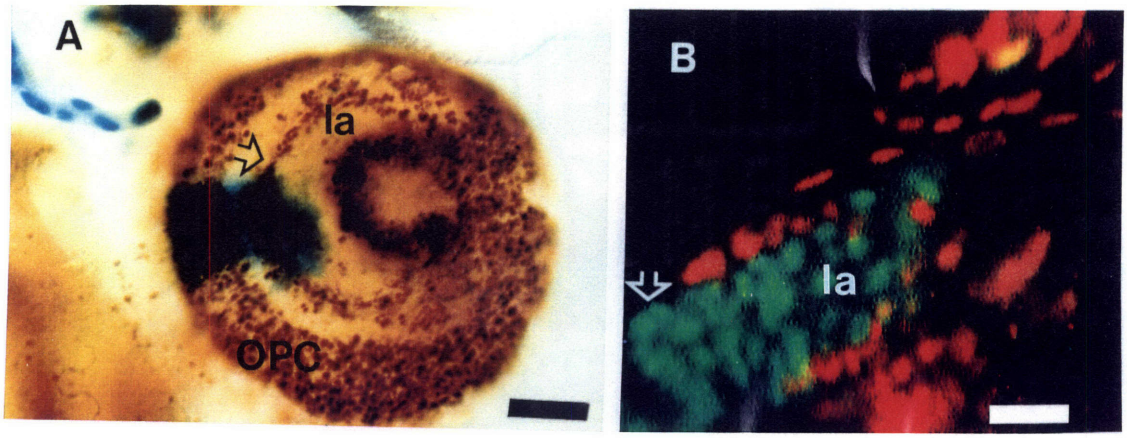
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Fig. 3. Analysis of L-glial mosaic clones.

Anterior is to the left in panels A-D, and towards the top of the page in panels E,F. In A-D, the mosaic clones were induced in first instar, or early second instar larvae. (A-C) Lateral views of lineage clones. In (A), the brain is pulse labeled with BrdU (brown) and co-stained with X-gal (blue) to detect the lineage clone. The lamina (la) and the outer proliferation center (opc) are indicated. An open arrow marks the position of lamina neurogenesis. (B,C) Adjacent sections through a single glial clone. The brain is stained with anti-ß-galactosidase (green) to detect the clone and co-stained with anti-RK2-5' (red) to detect glia. The satellite glial layer is shown in panel (B), while one of the deep L-glial layers is shown in (C). In each panel, a triangle marks the posterior border of the lamina, and indicates the dorsal/ventral midline of the tissue. (D) A clone is shown in horizontal view, and the brain is stained as in B,C. The three layers of lamina glia (satellite, s; epithelial, e; marginal, m) are indicated. An open arrow marks the position of lamina neurogenesis. In (E,F), single sections through two different glial patches are shown. The mosaic clones were induced in late second or early third instar larvae. The brains are stained as in B-D. Triangles mark the posterior border of the lamina. Scale bars: A, 12.5 μm; B and C, 10 μm; D, 23 μm; E, 7 μm; F, 17 μm.

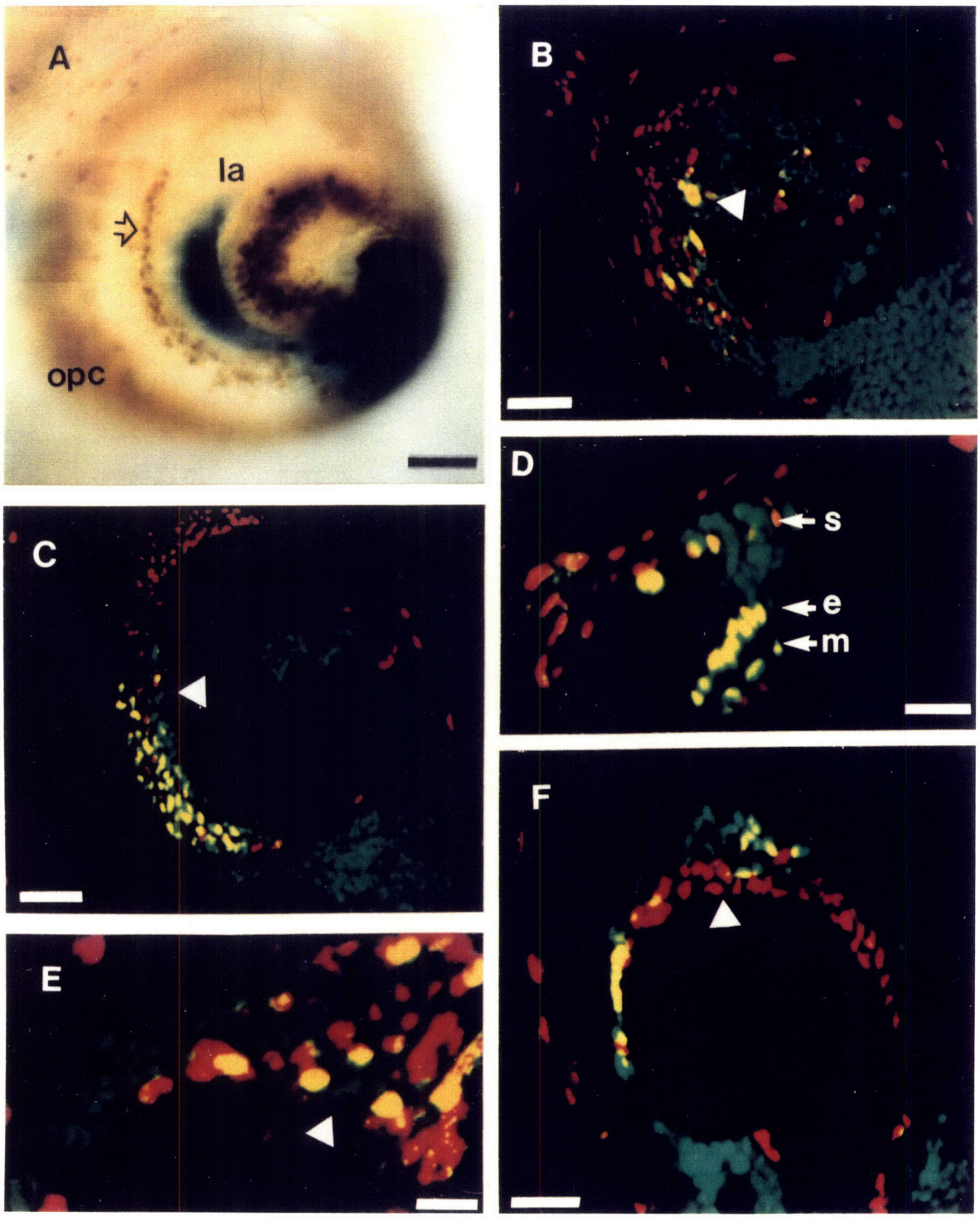
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Fig. 4. Mosaic clones in the subretinal and medulla glial layers.

Clones were induced during second or third instar, and examined in late third instar larvae. Brains are stained with anti-ß-galactosidase (green) to detect the lineage clone and co-stained with anti-RK2-5' (red) to detect glia. (A) Subretinal glia are related to glia in the optic stalk and in the eye disc. Anterior is to the left. The eye disc (ed), optic stalk (os) and subretinal glial layer (sr) are indicated. The optic stalk is outlined by white lines. An arrow indicates subretinal glia on the brain's surface. (B) Medulla glia arise from the ganglion's periphery. Anterior is to the top of the page. The two rows of medulla glia are indicated (me), as is the medulla neuropile (circle). Scale bars: A, 22 μm; B, 17 μm.

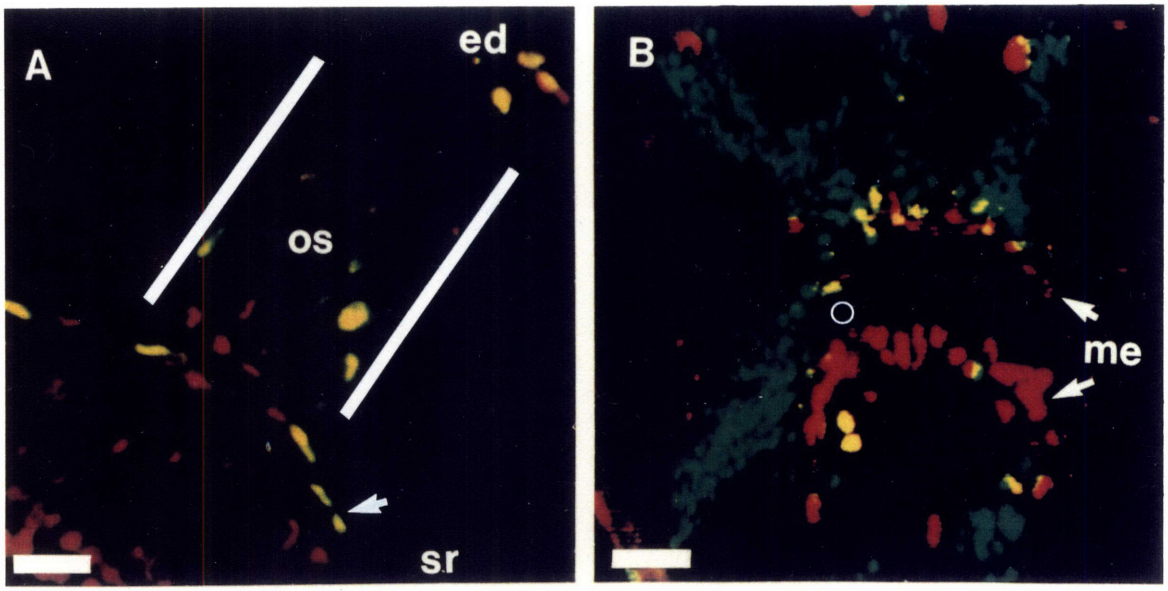
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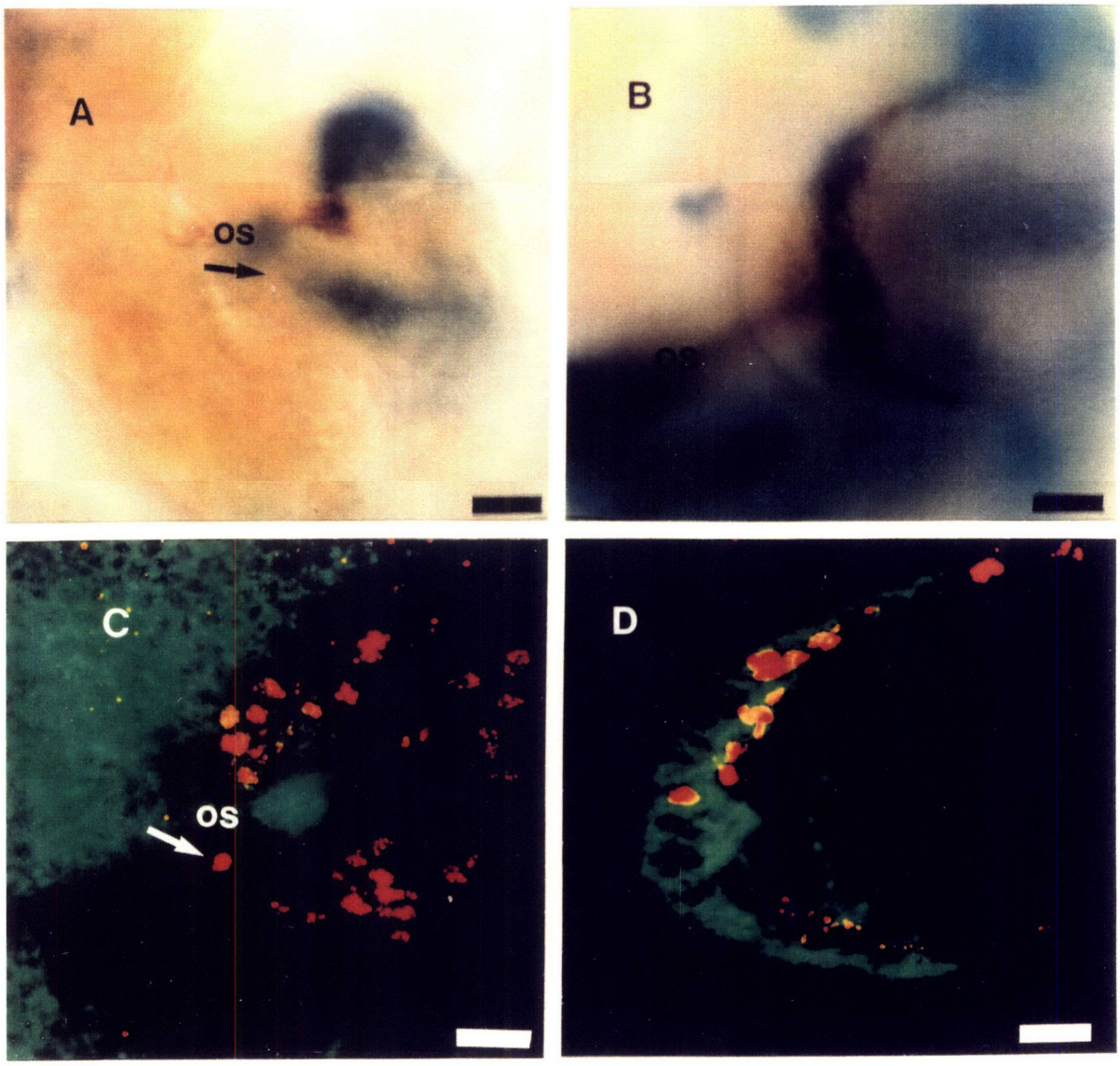
Fig. 5. Glia enter the lamina anlage prior to retinal input.

Clones in pre-innervation brains were induced during second instar, and analyzed during early third instar. Clones in well innervated brains were induced during second instar and analyzed during late third instar. All panels show the lamina in a lateral orientation. When it is in focus, the optic stalk is indicated (os). Arrows indicate the anterior border of pre-innervation clones. (A) pre-innervation and (B) post-innervation brains, double labeled with X-gal (blue) to detect the clone and MAb24B10 to detect photoreceptors (brown). (A) Clones in a preinnervation animal, between 69 and 72 hours AEL, are shown. (B) A clone in a well innervated animal is shown. (C) pre-innervation and (D) post-innervation brains, double labeled with anti-B-galactosidase (red) to detect the clone and anti-HRP (green) to detect photoreceptors. (C) A clone in a pre-innervation animal is shown. (B) A clone in a well innervated animal is shown. Scale bars: A, 9 μm; B, 10 μm; C, 5 μm; D, 9 μm.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B
OS
C
OS
D

Fig. 6. Distribution of glial cells in wild type animals.

All panels show lateral views of brains, stained with anti-RK2-5' (red) to detect glial nuclei and co-stained with anti-HRP (green) to detect R-axons. Anterior is to the top of the page in each panel. When appropriate, the optic stalk (os) is labeled. (A-D) Serial sections through a wild type third instar brain are shown. Each section is progressively more medial. Subretinal glia (sr) on the brain's surface are shown in (A). In (B), the satellite glial layer (s) is visible. Satellite glial nuclei are smaller than the subretinal glial nuclei. In (C), one of the two layers of deep L-glia is visible. An open arrow indicates the position of the trasmedullary fibers. In (D), the medulla neuropile is marked with an arrow, and the medulla glia which lie just beneath the lamina are visible (me). Scale bars: A-D, 12.5 μm.

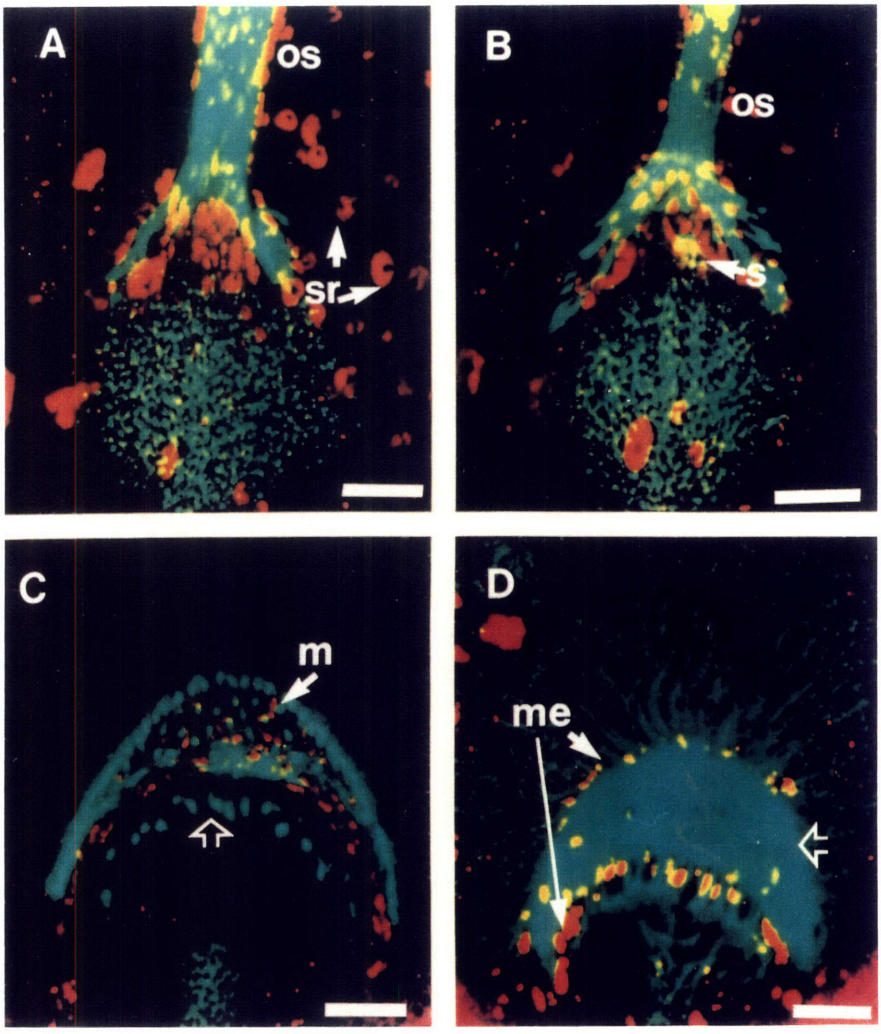
A
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Fig. 7. Distribution of glial cells in non-innervated animals.

All panels show lateral views of *sine oculis*^{1} brains, stained with anti-RK2-5' (red) to detect glial nuclei and co-stained with anti-HRP (green) to determine the extent of retinal innervation. The brains shown in panels (A-D) are non-innervated, while the brain shown in (E) is partially innervated. Anterior is to the top of the page in each panel. When appropriate, the optic stalk (os) is labeled. Panels A and B show glial layers that do not appear to be affected by the lack of retinal input, while panels C-E show glial layers that are not normally organized in the absence of retinal input. Subretinal glia (sr) are shown in (A), and the optic stalk (os) is indicated. In (B), the medulla neuropile is marked with an asterisk, and the medulla glia (me) are visible. (C,D) Two different layers of deep L-glia are shown. A section through an epithelial layer (e) is visible in (C), and one through a marginal layer (m) is visible in (D). The transmedullary fibers are marked with an open arrow. In (E), a section through the satellite glial layer of a partially innervated brain is shown. RK2 expression is restricted to the vicinity of the retinal axons (asterik). Scale bars: A-E, 12.5 μm.

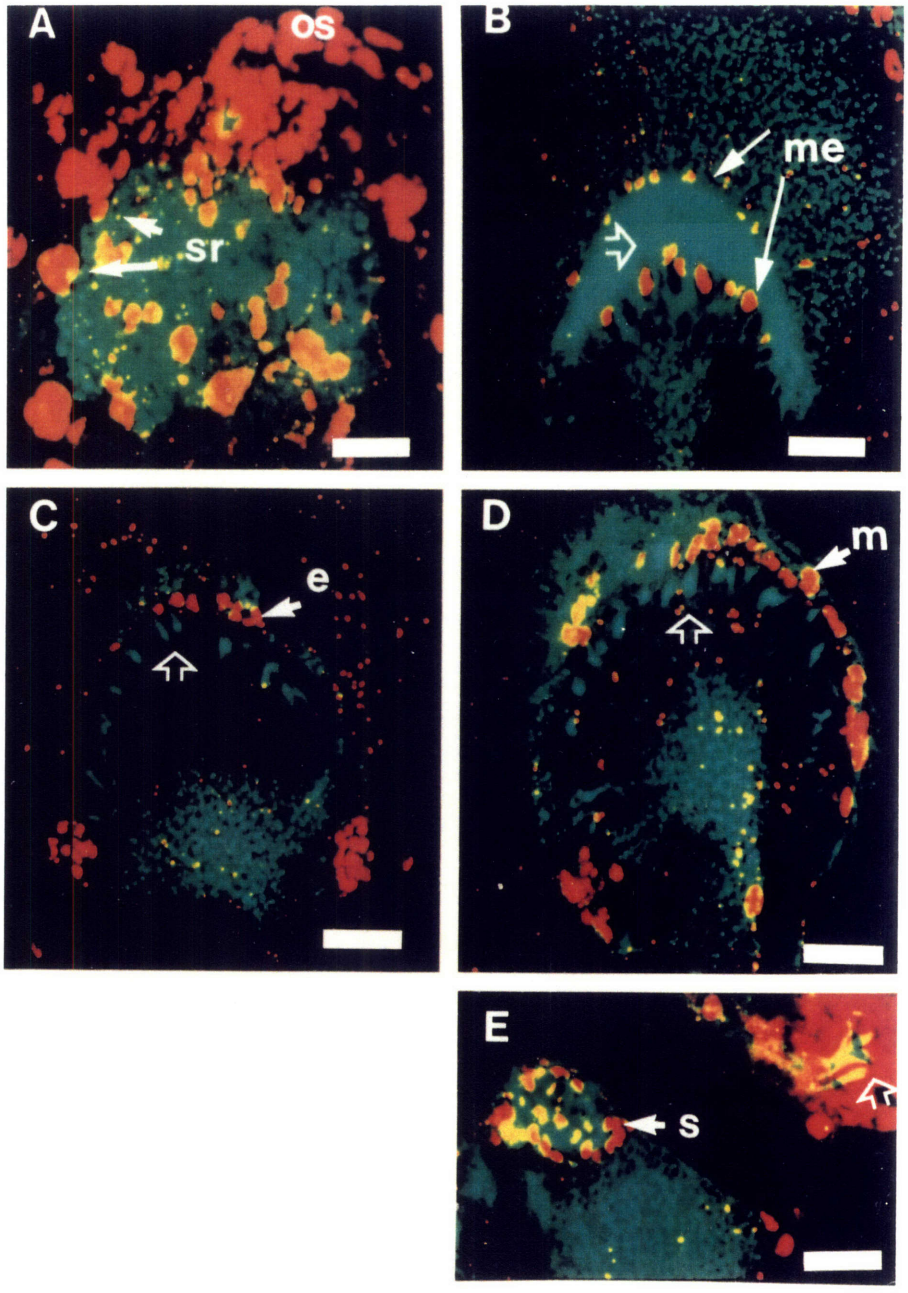
A
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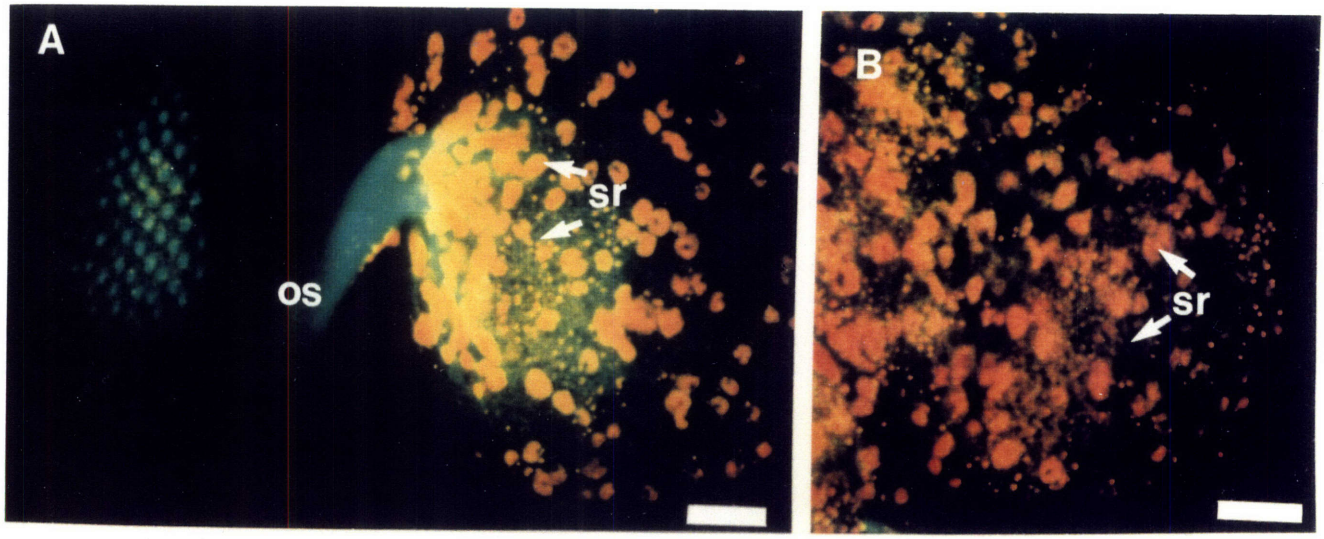
Fig. 8. Distribution of subretinal in *disconnected* animals.

Anterior is up in each panel. The brains are stained with anti-RK2-5' (red), to detect glia and co-stained with anti-HRP (green), to determine whether or not the brain is innervated. The lateral margin of the brain is to the left in panel (A), and to the right in panel (B). In (A), a wild type hemisphere is shown. The optic stalk (os) and subretinal glial layer (sr) are labeled. In (B), an "unconnected" *disco* hemisphere is show. The subretinal glial layer (sr) is indicated. Scale bars: A-B, 25 μm.


A
sr
os
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IV. Molecular and genetic analyses of *lama*, an evolutionarily conserved gene expressed in the precursors of the *Drosophila* first optic ganglion

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This chapter will be submitted for publication

SUMMARY

Drosophila retinal axons provide at least two developmental cues to their target field, which is called the first optic ganglion, or the lamina. Input from the eye triggers both the proliferation of lamina neurons and the terminal differentiation of the lamina glia. To date, the molecular basis of communication between the retinal axons and lamina progenitors has not been described.

We have used P element enhancer trapping to identify a new gene, *lamina ancestor* (*lama*). *lama* is expressed by both the neuronal and glial precursors of the first optic ganglion, despite the fact that these cells come from distinct lineages. The gene's expression pattern is striking because it correlates with a specific developmental stage of the lamina's progenitors. *lama* is expressed by the L-neuron precursors as they are completing their final divisions, and by the L-glial precursors until they begin their migration into the lamina anlage. In both cases, *lama's* expression in the precursor cell is down regulated once the cell responds to retinal input and is incorporated into the ganglion.

We examined the gene's expression pattern early in development, and in the absence of retinal innervation. We found that the glial precursors begin to express *lama* as early as first instar, while expression in the neuronal precursors begins over two days later, coincident with input from the eye. In the absence of axon ingrowth, both classes of precursors express *lama* and accumulate on the brain's surface. This data is consistent with the gene's involvment in either progenitor response to retinal input, or in the specification of a "lamina" specific fate. A null mutation was generated in *lama*, and homozygous mutants are viable and fertile. The visual system appears to develop normally in these animals. Nevertheless, we believe that *lama* encodes an important function. The *D. melanogaster* ORF encodes a novel protein that is 74% identical to *Drosophila virilis* lama, indicating that the gene is under selective pressure.

INTRODUCTION

Studies of the *Drosophila* visual system have added immeasurably to our understanding of basic developmental mechanisms. Analyzing retinal

morphogenesis has illuminated how pattern emerges in an undifferentiated epithelium (see Ready, Hanson and Benzer, 1976; and reviewed in Wolff and Ready, 1991 and Heberlein and Moses, 1995). Investigations of ommatidia formation have yielded a wealth of information about how cell-cell interactions affect fate choices (reviewed in Hafen and Basler, 1991 and Zipursky and Rubin, 1994). In addition, the *Drosophila* visual system has provided a setting in which some of the fundamental questions in neurobiology are genetically tractable. For example, the issues of axon guidance (Martin, et. al., 1995; Schneider et. al., 1995), neural-glial interactions (Xiong and Montell, 1995), and retrograde trophic support (Campos et. al., 1992) have begun to be addressed. An area less well explored is that of how retinal innervation of the brain affects the development of the first optic ganglion, the lamina.

Lamina development is coordinated with retinal innervation of the brain, which begins midway through third instar larval life (Meinertzhagen, 1973). Early work by Power indicated that the lamina's size correlates with the size of the retina (Power, 1943). Genetic mosaic analyses demonstrated that some mutations which act within the retina to disrupt its development (Meyerowitz and Kankel, 1978; Fischbach and Technau, 1984) also perturb lamina formation. Many of the cellular interactions between the developing eye and brain have been described. As retinal axons grow into the brain, they induce the proliferation of the lamina's neuronal progenitors (Selleck and Steller, 1991). Input from the eye also triggers the terminal differentiation of the lamina glial cells (Winberg, et. al., 1992), as well as their complete migration into the optic anlage (Perez and Steller, submitted).

The molecular basis of communication between retinal axons and cells in the developing brain has not been described. We screened P element enhancer trap lines in order to identify those which showed reporter expression in the retinal target field. We were particularly interested in isolating lines which showed expression in the lamina precursors. The lamina neuronal (L-neuron) and glial (L-glial) precursors arise from distinct lineages, and respond very differently to retinal input (Winberg et. al., 1992; Perez and Steller, submitted). Despite these differences, we identified a novel gene expressed by both classes of precursors. In late third instar animals, the

gene is expressed in neuronal and glial precursors which surround the lamina, and is abruptly down regulated once the precursors are incorporated into the developing ganglion.
We examined the gene's expression pattern at earlier stages, and found that it is expressed in the L-glial progenitors as early as the first instar. Expression in the neuronal precursors begins 60 hours later, following input from the eye. In the absence of innervation, both classes of precursors express the gene and remain on the brain's surface, indicating that axonal cues are necessary for the organization of the lamina's multiple cell layers. We have named this locus *lamina ancestor*, or, *lama*.

lama encodes a novel ORF of 624 amino acids. We generated a null mutation in *lama*, and found that it does not appear to disrupt the development of the visual system. Widespread, ectopic expression of *lama* is similarly innocuous. Nevertheless, we believe that *lama* is likely to encode an important function for the following reason. Despite the 60 million years of divergence between *D. melanogaster* and *D. virilis* (Beverly and Wilson, 1984), the lama protein is 74% identical between these two fly species. Furthermore, *lama* is expressed in some of the same cell types in *D. virilis* as it is in *D. melanogaster*. Other genes required for visual system development are not as well conserved (Heberlein and Rubin, 1990; Michael et. al., 1990). Our data are consistent with *lama* playing a role in either the specification of a "lamina" identity or in the response of lamina precursors to retinal input.

MATERIALS AND METHODS

Stocks and genetics

A P element enhancer trap screen (Bellen, et. al., 1989; Bier, et. al., 1989) was carried out in our lab, and in the 1990 M.I.T. project lab. A description of the screen has been previously reported (Winberg et. al., 1992). ry^{506} is the parental strain of our P element lines, and the PZ enhancer trap construct was used (Mlodzik and Hiromi, 1992). We screened for lines which showed reporter expression in the developing optic lobes. Two viable insertions, PZA5 and PZA8, were identified. An additional insertion, PlArbL733, was the gift of R. Davis. Each insertion shows an identical expression pattern in the lamina precursors. To determine whether reporter expression requires retinal input, the reporters were crossed into two mutant backgrounds, *eyes*

absent^1 (*eya*^{1}) (Bonini et. al., 1993) and *sine oculis*^{1} (*so*^{1}) (Fischbach and Technau, 1984; Cheyette et. al., 1994). Additionally, reporter expression was examined in embryos, and in young larvae, prior to innervation of the brain. The developmental stages and structures of the embryo were determined by a comparison to Hartenstein and Campos-Ortega (1985). Larvae were staged from egg deposition, and ages are given as hours after egg laying (AEL).

A P element excision mutagenesis was carried out by crossing the reporter lines to a source of the P transposase. In one P element excision scheme, we crossed the P element reporter to the Δ2.3 chromosome (Laski, et. al, 1986; Robertson, et. al., 1988). Males of the Δ2.3/PZA8 genotype were collected and crossed to mwh, red, e/TM3Sbry^{RK}. Progeny were scored for loss of the ry$^+$ marker, and ry$^-$ animals were crossed to CxD/TM3Sb animals to establish excision lines. In an alternative screen, we used a source of transposase on the X chromosome, Hop6yw, provided by W. Gelbart. The P element reporter was excised across from a chromosome bearing a deficiency for the region, Df(3L) ems-13 (gift of J. Fristrom), which uncovers 64B-E. Males of the genotype Hop6yw; PZA8/Df ems-13 were crossed to mwh, red, e/TM3Sbry^{RK} females. Excision lines were established as described above.

In situ hybridization to polytene chromosomes

P element insertions were mapped via *in situ* hybridization to polytene chromosomes, using a modification of the technique described by M-L. Pardue (1994). pBluescript (Stratagene) was used to detect the *lacZ* sequences within each P element vector. An additional insertion, 3-0403, was mapped by the *Drosophila* Genome Center.

Isolation of genomic clones surrounding the P element insertions

Standard molecular biological protocols were used (Sambrook et. al., 1989). Genomic DNA surrounding the site of the insertions was obtained by plasmid rescue (Steller and Pirotta, 1986) from the PlArbL733 line. The structure of PlArb has been previously described (Wilson et. al., 1989). We recovered 4.5 kB of flanking DNA. The rescued plasmid was used to screen a library of Canton S genomic DNA in the lambda dash II vector (gift of R. Davis). 30 kB in overlapping phage were isolated. The rescued plasmid

hybridized to a 5.5 kB R1H3 genomic fragment, which was subcloned into pBluescript II KS+ (Stratagene) to generate pB11-5.5. The PZA5 and PZA8 insertions were mapped within the walk to the same 5.5 kB fragment by a combination of Southern analysis and PCR. The cytological location of the genomic DNA was verified by using one of the lambda clones as a probe against a Canton S polytene squash (see above).

To identify putative transcription units adjacent to the P element insertions, regions of the walk were used to probe *Drosophila virilis* Southerns. Because *D. melanogaster* and *D. virilis* are diverged by 60 million years (Beverly and Wilson, 1984), sequences conserved between the two fly species are likely to be within transcription units. Genomic DNA was prepared from *D. virilis* flies as described previously (Steller and Pirotta, 1986). Southern analysis was performed at low stringency (42°; 25% formamide). In addition to the 5.5 kB insertion fragment, two fragments (a 4.0 kB R1H3 fragment and a 6.6 kB R1 fragment) cross-hybridized to *D. virilis*, and were subcloned into pBluescript, to generate pB11-4.0 and pB14-6.6.

Isolation and characterization of cDNAs

A third instar larval (3IL) CNS cDNA library, generated by C. Wilson (Wilson et. al., 1993), was screened with the 4.0 kB R1H3 insertion in pB11-4.0. A size selected, 9-11 hour embryonic cDNA library was provided by K. Zinn (Zinn et. al., 1988), and was screened with the 5.5 kB R1H3 insertion in pB11-5.5. Two distinct classes of cDNAs were isolated, and the longest clone of each class was chosen for further analysis. cDNAs were subcloned from the lambda vectors into pBluescript. A 1.1 kB Spe1 fragment, representing part of the 2.7 kB 3IL cDNA, was subcloned into pBluescript to generate pB5s1-1.1. A 3.25 kB R1 fragment, representing the entire embryonic cDNA, was subcloned into pBluescript to generate pBe2.

DNA sequencing and analysis

All sequencing was done using Sequenase (United States Biochemical). The pB5s1-1.1 and pBe2 clones were sequenced using a combination of restriction deletions and primer walking. Partial sequence obtained from the pB5s1-1.1 insertion indicated that it corresponded to a previously published kinesin

homologue, KLP64D (Stewart et. al., 1991). Both strands of the pBe2 insertion were sequenced. Within the corresponding genomic clones (pB11-5.5, and pB14-6.6), both strands of the coding sequence, six of the eight intron/exon boundaries, and substantial portions of the 5' and 3' UTRs were also sequenced. This locus encodes a novel protein, *lama*. Sequence analysis was performed using the Genetics Computer Group program.

Northern analysis

Each of the cDNAs was used to probe Northern blots of total RNA isolated from 3IL CNS tissue using a modification of a previously described protocol (Chirgwin et. al., 1979). Total RNA from approximately 20 dissections was loaded in each lane. Genomic DNA covering *lama's* 5' exon was also used to screen a Northern blot of embryonic RNA (6 ug, poly-A selected).

***In situ* hybridization to whole mount tissue**

The expression patterns of the transcripts were determined by whole mount *in situ* hybridization (Tautz and Pfeifle, 1989). Two digoxigenin (Boehringer Mannheim) labeled probes were made from pB5s1-1.1. A riboprobe, and a random primed DNA probe, were synthezied using protocols from the manufacturer. Third instar larval (3IL) CNS expression of KLP64D was determined as previously described, using the random primed digoxigenin probe (Tautz and Pfeifle, 1989). The riboprobe was subjected to base-hydrolysis, and used to determine the KLP64D embryonic pattern (see Appendix 1). The hybridization conditions have been previously described (Grether, et. al., 1995). To determine the expression pattern of the *lama* cDNA, a digoxigenin labeled riboprobe was made from the pBe2 cDNA. *lama's* expression pattern in embryos was determined as described above. *lama's* expression pattern in the 3IL CNS was determined using a modification of a previously described protocol (Zak and Shilo, 1992). Following hybridization of the probe and subsequent washes, the tissue was incubated in anti-digoxigenin antibody (Boeringer Mannheim) at 1/4000 in PBS + 0.1% tween (PBT) for 1 hour at room temperature. The anti-digoxigenin antibody was coupled to an alkaline phosphate moiety, and had been preabsorbed at 1/100 against fixed embryos. *lama's* expression pattern in the ovaries was determined as previously described (Ephrussi et. al., 1991).

PCR screen for lama mutations

To identify *lama* mutations following a P element excision mutagenesis, genomic DNA prepared from the excision lines (Steller and Pirotta, 1986) was screened via PCR (Saiki, 1990). Five combinations of PCR primers were used to amplify products from the genomic DNA templates. The first basepair of the cDNA is designated as +1 (Fig. 7). The primers amplified the following sequences: -900 to +70, -900 to +380, -100 to +70, +20 to +350, +100 to +350, +210 to +350, and +1780 to +1885. All potential excision mutations were verified by Southern analysis.

Ectopic expression analysis

The 3.25 kB *lama* cDNA was cloned into the R1 sites within the pCaSper-hsp70 construct (Grether et. al., 1995), to generate P[*hs-lama*; w^+]. The orientation of insertion was determined by restriction analysis and sequencing. Germline transformation was performed as previously described (Grether et. al., 1995). 1800 *yw* embryos were injected, and one transformant was obtained. To verify that the construct provides ectopic *lama* expression upon heat shock, wild type third instar larvae (3IL), and 3IL carrying a single copy of the transgene, were subjected to a 1 hour heat pulse of 39°. The brains were dissected and analyzed by whole mount *in situ*, as described above. For the developmental analysis, 12 hour collections of wild type embryos, and collections of embryos carrying 1-2 copies of the transgene, were taken and aged to first instar. The animals were reared at room temperature, and subjected to a 1 hour, 37° heat pulse, once a day until late 3IL. Repetitive 37° heat pulses were used instead of 39° heat pulses, because neither the wild type nor transformed collections survived multiple 39° exposures. The brains were dissected from late 3IL following the heat shock regime, and analyzed for developmental abnormalities with respect to cell proliferation, retinal innervation of the target field, and neuronal and glial development as described below.

Immunohistochemistry

All antibody staining was carried out as previously described (Winberg et. al., 1992). For X-gal staining, the larval central nervous system (CNS) was

dissected in 0.1M NaPO4, pH 7.2, and fixed for 1 min. in 0.5% glutaraldehyde in 0.1M NaPO4, pH 7.2. The CNS was then incubated at 37° for 1-8 hours in X-gal (Bachem) stain solution as previously described (see Bier et. al. ,1989). The tissue was rinsed in PBS + 0.3% triton (PBT), and mounted in 70% glycerol for viewing. For X-gal/BrdU double labeling, the brains were pulse labeled *in vitro* with BrdU. The animals were dissected in Grace's media (Gibco BRL) containing BrdU (Boerhinger Mannheim) at a final concentration of 30 μg/ml. Labeling was allowed to continue for 30 minutes at room temperature, and the brains were then fixed for 30 minutes in 2% paraformaldehyde (Polsyciences, Inc.) in 0.1 M Phosphate buffer, pH 7.2-7.4. The brains were rinsed in PBT and stained with X-gal. Following X-gal detection, the tissue was processed for BrdU detection by incubating it in 2N HCl in PBT for 20 minutes at room temperature, which exposes the BrdU epitope. Larvae were dissected in 0.1M phosphate buffer (pH 7.2-7.4) and the brain hemispheres were fixed for 30 minutes in 2% paraformaldehyde at room temperature. Adult heads were mounted in OCT (Tissue Tek), and 10-12 μm frozen sections were cut on a Frigocut cryostat. Sections were post fixed for 30 min. in 2% paraformaldehyde. Following immunohistochemistry, sections were counter stained with bisbenzimide to detect nuclei (Ashburner, 1989). For staining with Mab44C11 (Bier et. al., 1988), the tissue was treated with collagenase (0.3 units/microliter) (Sigma) for 20 minutes at room temperature prior to fixation, to allow access of the antibody. A 100x collagenase stock (in 50% glycerol, 40 mM $NaPO_4$ buffer, pH 7.4) was diluted in 0.1M $NaPO_4$, pH 7.2, prior to use. The following antibodies were used at a dilution of 1/100-1/200: mouse anti-BrdU (Becton Dickinson), rabbit anti-β-galactosidase (Cappel), goat anti-mouse-HRP (BioRad), goat anti-rabbit-FITC (Cappel), goat anti-rat-Cy3 (Jackson Immuno) and anti-RK2-5' (Campbell, et. al., 1994). Mab24B10 (Zipursky et. al, 1986) and MAb44C11 (Bier et. al, 1988) was used at 1/5. The anti-RK2-5' antibody was the gift of A. Tomlinson. Mab24B10 was a gift from S. Benzer, and MAb44C11 was a gift from Y.N. and L. Jan. Samples were viewed on a Zeiss Axiophot. Immunofluorescence was visualized with a Biorad confocal microscope using software provided by the manufacturer.

Histology

Adult heads from 7-10 day old *ry*^{506} and *lama*^{410} animals were embedded in Spurrs media (Spurr, 1969). Adult heads from animals carrying the hs-lama transgene were also embedded. The animals had been subjected to a series of heat shocks (see above) throughout larval and pupal life, and aged for 2-5 days prior to head dissection. Semithin (1 μm) sections were taken through the middle third of each sample. Sections were stained for 2 minutes at 50° in .05% toluidine/.01%methylene blue in .05% sodium borate, rinsed in distilled H_2O, and dried. Sections were mounted under DPX (Fluka), and viewed on a Zeiss Axiophot.

Isolation and characterization of a *D. virilis* homologue

A *Drosophila virilis* genomic library in the lambda EMBL3 vector was provided by J. Tamkun. The library was plated and screened at low stringency (42°; 20% formamide) using standard protocols. A 1.18 kB ApaHincII fragment from pBe2 (corresponding to residues 56-542 in the ORF) was used as a probe. One phage was isolated, and the region of homology was delineated. The sequence of the *D. virilis* homologue and its expression pattern were determined as described above.

RESULTS

Identification of a gene expressed in the lamina precursors

We identified 3 P element insertion lines, mapping to 64C11 (Fig. 1A), which show β–galactosidase reporter expression in the lamina precursors. A schematic of the developing lamina (la) is shown in Fig. 1B. The dorsal/ventral and anterior/posterior axes are labeled. The lamina develops progressively, such that its oldest cells are found at its posterior border (Meinertzhagen, 1973). At this stage, the newest retinal axons, and the most recently incorporated neurons and glia, are found at the lamina's anterior edge (White and Kankel, 1978; Selleck and Steller, 1991; Perez and Steller, submitted). The lamina neurons (L-neurons) are generated by a population of dividing cells located at the anterior border of the tissue, which are designated by solid circles in Fig. 1B. Lamina glia (L-glia) arise from the dorsal and ventral posterior corners of the lamina, which we refer to as the glial

precursor zones (shown in Fig. 1B by stippled circles; Perez and Steller, submitted). The direction of precursor migration is indicated by arrows in Fig. 1B.

In late third instar larval brains, the strongest expression of the β–galactosidase reporter is seen in neuronal precursors at the anterior border of the lamina (arrowhead; Fig. 1C), and in cells located in the glial precursor zones (arrows; Fig. 1D). Expression overlaps the final divisions of the neuronal precursors (arrowhead; Fig. 1E). In Fig. 1E, reporter expression is shown via an X-gal stain (blue), and cell proliferation is detected via BrdU (brown). Reporter expression is seen in the glial precursor zones, but is not seen in the layers of mature L-glia (arrows). In Fig. 1F, the reporter is detected via anti-β–galactosidase (green), and the glia are detected with a glial antibody, anti-RK2-5' (red) (Campbell et. al., 1994).

Once cells have been incorporated into the lamina, they are organized into distinct layers which are visible in cross sections through the tissue. A schematic diagram of a cross section through the lamina (la) is shown in Fig. 1G. The anterior/posterior axis is indicated, and one retinal axon is drawn. The cell bodies of the L-neurons lie above the L-glial layers. The anterior border of the lamina is marked by a depression in the brain's surface, called the furrow, which is indicated by filled ovals in Fig. 1B. The newest retinal axons abut the furrow, and L-neurons emerge into the lamina from its posterior wall (White and Kankel, 1978; Selleck and Steller, 1991; Selleck et. al., 1992).

β–galactosidase reporter expression is contiguous around the furrow, which is approximately 4 cells deep, and is not seen in cells within the body of the lamina (Fig. 1H). Thus, it appears that the corresponding gene is down-regulated upon incorporation of the precursors into the developing ganglion. In Fig. 1H, reporter expression is detected via anti-β–galactosidase (green), and retinal axons are detected via a general neuronal marker, anti-HRP (red) (Jan and Jan, 1982). An arrowhead indicates the furrow's posterior wall.

Expression of the β–galactosidase reporter corresponds to the expression of a novel gene (see below). Because the gene is expressed by both the lamina's neuronal and glial precursors, we have named this locus *lamina ancestor*, or, *lama*.

lama expression is independent of retinal innervation

We investigated whether *lama* expression in the lamina progenitors preceeds input from the eye. If *lama* is involved in the brain's response to axon ingrowth, its expression should be independent of retinal innervation, which begins at approximately 94 hours AEL.

In first instar larvae, at approximately 24-26 hours AEL (Fig. 2A), the strongest *lama* expression is seen in two, symmetrical zones on the surface of each brain hemisphere. The zones are located in the ventral-posterior region of the hemispheres, in a location that is consistent with expression in the optic lobe anlage (Hofbauer and Campos-Ortega, 1990). Expression of *lama* continues throughout larval life, and the symmetrical zones show a substantial increase in size between the second and third instar stages (Fig. 2B-D; approximately 50, 90 and 94 hours AEL). These zones appear to lie on the brain's surface at the base of the eye stalk, and are likely to correspond to the glial precursor zones found at the dorsal and ventral posterior corners of the lamina in late third instar animals (Perez and Steller, submitted; and see Fig 1B).

Expression is seen in the eye disc of early third instar larvae (Fig. 2C), and within the photoreceptors at the onset of retinal innervation of the brain (Fig. 2E,F; approximately 94 and 102 hours AEL). Transient expression is also seen in L-glia within the lamina (Fig. 2D,F). In well innervated brains, *lama* is expressed in neither photoreceptors nor L-glia within the lamina (Fig. 1E,F).

As innervation proceeds, fewer cells within the glial precursor zones express *lama* (compare Fig. 1D to Fig. 2D,F). The decrease in the size of the glial precursor zones correlates with glial migration into the body of the lamina (Perez and Steller, submitted). This expression pattern is consistent with an early, and sustained, expression of *lama* in the L-glial progenitors, and with the downregulation of *lama* once an immature glial cell begins its migration into the lamina.

Expression of *lama* in the neuronal precursors begins in third instar life, and appears to coincide with retinal innervation of the brain. Following retinal input, at approximately 94 hours AEL, expression is restricted to rows of cells which lie perpendicular to the anterior border of the lamina (Fig. 2D,E). Expression overlaps with a population of dividing cells (Fig. 2D),

which are likely to correspond to the lamina neuronal precursors. In Fig. 2D, *lama* expression is detected via an X-gal stain (blue), and cell proliferation is detected via BrdU (brown).

As is the case in well innervated brains, *lama* is expressed in neuronal precursors that are adjacent to retinal axons (Fig. 2E). In Fig. 2E, *lama* is detected via anti-βgalactosidase (green) and retinal axons are detected via a photoreceptor specific antibody, MAb24B10 (red) (Zipursky et. al., 1984). In slightly older animals, expression is seen in clusters of neuronal precursors anterior to the lamina (Fig. 2F; approximately 102 hours AEL). By late 3IL, the brain is well innervated, and cells expressing the reporter at the lamina's anterior are arranged into two rows which surround a furrow at the lamina's anterior border (see Fig. 1C,H).

In order to determine whether *lama* expression in the neuronal precursors is induced by retinal input, we examined the gene's expression pattern in *sine oculis*^{1} (Cheyette, et. al., 1994) and *eyes absent*^{1} (Bonini, N.M. et. al., 1993) mutant backgrounds. Each mutation disrupts retinal development, and severely reduces (*so*^{1}), or eliminates (*eya*^{1}), innervation of the brain. The *so*^{1} mutation has been demonstrated to act autonomously in the eye (Fischbach and Technau, 1984). Consequently, any variation in reporter expression within the optic lobe, between wild type and *so*^{1} animals, may be attributed to the lack of input from the eye. In the experiments described below, *lama* expression in late 3IL *so*^{1} brains is detected via an X-gal stain (n=84). Similar experiments were done in which the reporter was detected with anti-βgalactosidase, and in these cases the extent of retinal innervation of the brain was determined with the general neuronal marker, anti-HRP (Jan and Jan, 1982) (n=53; data not shown).

In partially innervated, or non-innervated, *so*^{1} animals, the glial precursor zones, indicated by arrows in Fig. 3A, are consistently larger than they are in similarly aged wild type brains (compare to Fig. 1D). Expression resembles that seen in a newly innervated brain (Fig. 2D,F), in that the glial precursor zones are larger than they are in a well innervated brain. Expression is also seen in the neuronal precursors, which are found in clusters on the brain's surface and lack the organization that is found in well innervated brains (arrowhead, Fig. 3A and compare to Fig. 1C).

In the absence of input from the eye, *lama* expression is weaker, and more

variable, in the neuronal precursors than it is in the glial precursors. In 85% of the non-innervated, so^1 brain hemispheres, the distribution of neuronal precursors, as assayed by *lama* expression, appears to be extended along the anterior/posterior axis relative to their distribution in wild type brains (compare Fig. 3A to Fig. 1C).

The expression of *lama* in *eya* animals resembles the expression seen in so^1 animals (Fig. 3B). In both mutants, the precursors remain on the brain's surface, and *lama* expression is not down regulated as it is in wild type animals. Thus, the onset of *lama* expression in both the L-glia and L-neuron precursors is independent of retinal input, but its modulation requires retinal cues. Based on its expression pattern, *lama* was viewed as a good candidate for a gene involved in either the specification of lamina identity, or in the communication between ingrowing R-axons and the lamina precursors.

Molecular characterization of the *lama* region

We were interested in cloning the gene expressed in the same pattern that is shown by the β–galactosidase reporters. Genomic DNA adjacent to the P element insertions was isolated as described in the Materials and Methods. The PZA8, PZA5, and PlArbL733 insertions were mapped and found to be inserted within approximately 1 kB of each other (Fig. 4). We identified areas adjacent to the P element insertions that were likely to be transcribed (see Materials and Methods), and used the corresponding genomic clones to screen cDNA libraries. Two classes of cDNAs were isolated, and the longest clones were chosen for analysis. Each cDNA detects a single transcript upon Northern blot analysis. The expression pattern of each transcript was determined by using each cDNA as a probe for whole mount *in situ* hybridizations (see below). Only one of the transcripts is expressed in the developing adult visual system.

The expression of the *lama* cDNA matches the P element reporter expression at all stages examined (data not shown). The P elements are inserted 5' to *lama* (Fig. 4). The 5' end of the 3.25 kB cDNA lies 398 base pairs from the nearest P insertion, PZA8. Northern blot analysis indicated that the cDNA detects a single, 3.5 kB transcript in 3IL, and that a probe covering *lama's* first exon detects a single transcript of the same size in the embryo (data not shown). There is a consensus TATA box within the genomic clone

located 83 bp 5' to the start of the cDNA. Because TATA boxes are generally found 20-30 bp 5' to transcription start sites (Darnell et. al., 1986), we believe that the cDNA is missing approximately 50 bp at its 5' end. The *lama* ORF (Fig. 4, solid rectangle) is contained in the last two of *lama's* five exons.

Sequence of the other cDNA identified it as a member of the kinesin family, KLP64D (Stewart et. al., 1991). The P elements are inserted 3' to KLP64D, and the 2.7 kB cDNA detects a single, 2.9 kB transcript in 3IL. This agrees reasonably well with the size of the embryonic transcript that was previously reported (Stewart, et. al., 1991). Expression of KLP64D is seen in both the embryonic PNS and CNS, but is not seen in the developing 3IL visual system (Perez and Steller, unpublished; see Appendix 1). Therefore, we believe that the 64C11 P element reporter expression corresponds only to expression of the *lama* cDNA.

Developmental expression of *lama*

We examined the distribution of *lama* mRNA using whole mount *in situ* hybridization. During embryogenesis, *lama* is seen at 3 hours AEL in the region of the invaginating cephalic furrow (Hartenstein and Campos-Ortega, 1985), and in a single stripe at the posterior of the embryo (Fig. 5A). Between 5-7 hours AEL, expression is seen in the developing brain (arrowhead), and in a segmentally repeated fashion within the ventral nerve cord (Fig. 5B). The ventral nerve cord expression is found to either side of the midline (arrow) (Fig. 5C). In older embryos, *lama* is most strongly expressed in the brain hemispheres (arrowhead) and in the gonadal primordium (arrow) (Fig. 5D).

Post-embryonically, *lama* expression is seen most strongly in the lamina neuronal and glial precursors (Fig. 6A, and see Figs. 1,2). In adult animals, expression is seen in the ovarian follicle cells (Fig. 6B). We also examined expression via X-gal staining, and found that *lama* is expressed in testicular pigment cells, and in the subretinal glial cells of the adult head (data not shown).

Sequence analysis of *lama*

The longest ORF within the *lama* cDNA encodes a protein of 624 amino acids with a predicted molecular weight of 69 kD (Fig. 7). The ORF agrees well with *Drosophila* codon usage. There are two, in frame methionines (bp 991 and

1216), which agree equally well with the consensus start sequence for *Drosophila* (Cavener, 1987). At each methionine, five of the seven bases match the C/A-A-A-A/C-A-T-G consensus. There are multiple stop codons in all three reading frames 5' to the presumptive initiator methionine (bp 991) and 3' to the stop codon (bp 2865). Intron-exon boundaries are indicated in bold. All of the analyzed splice donor and acceptor sites correspond to the consensus sequences (Darnell et. al., 1986). A poly-adenylation consensus site (Birnsteil, et. al., 1985; bold and underlined) is found 355 bp 3' to the stop codon. A Chou-Fasman prediction of secondary structure indicates that the ORF is not likely to encode either significant stretches of α-helices or β-sheets. The protein is mainly hydrophilic, and has a predicted isoelectric point of 5.4. The are 3 consensus N-linked glycosylation sites, which are indicated with open circles, and eight potential targets for phosphorylation by cAMP kinase (underlined) (Creighton, 1984). Within the ORF, five base pair differences were found between the genomic clone and the cDNA . All but one of the changes was silent. The genomic clone codes for a lysine instead of a methionine at residue 474.

The sequence of the lama protein was compared to sequences in the GenBank, EMBL, SBASE (Pongor et. al., 1994), PROSITE (Bairoch, 1992) and SWISS-PROT (Bairoch and Boeckmann, 1992) databases. Comparisons were performed using the BLAST (Altschul et. al., 1990), FASTA (Pearson, 1990), BLOCKS (Henikoff et. al., 1994) and BEAUTY (Kim et. al., 1995) sequence comparison programs. No significant similarity is seen between lama and protein sequences within the databases. However, two regions of lama, residues 45-82 (motif I) and 598-622 (motif II), appear to be conserved in *C. elegans*. Partial sequence of several cDNAs indicate that motif I is found in 2 *C. elegans* genes, and is 39-45% identical at the amino acid level to the *Drosophila* sequence (Waterston et. al., 1992; accession numbers Genbank: M75762; EMBL: Z14766). Motif II appears in 1 *C. elegans* genes, and is 52% identical to the *Drosophila* sequence (dbj accession numbers D33409). The alignments between the motifs in lama and the conceptual translations of the *C. elegans* cDNAs is shown are figure 8.

Genetic analysis of 64C11

We were interested in examining the effects of a *lama* mutation on the development of the visual system, and therefore examined the gene's expression in animals homozygous for the P element insertions in 64C11 (data not shown). Because the insertions do not disrupt *lama* expression, we carried out a P element excision mutagenesis (n=600). We identified one lethal complementation group in the region which appears to correspond to mutations in KLP64D. All of the lethal excisions delete into the KL64D locus (Fig. 9). Each KLP64D allele removes a H3 site (indicated by an asterisk in Fig. 9), which lies 780 bp 3' to the KLP64D transcript. Whole mount *in situ* hybridization was used to determine whether KLP64D alleles affect the expression of *lama*. The lethal excisions do not affect the expression of *lama*, nor do small deletions confined to the intragenic region (data not shown).

Five *lama* mutations were identified by molecularly screening excision chromosomes, as described in the Materials and Methods. Animals homozygous for all of the alleles, including the null allele, $lama^{410}$, are viable and fertile (Fig. 9). Four of the five mutations break in *lama's* first exon, or within its first intron. We examined the effects of two of these small deletions ($lama^{607}$, $lama^{615}$) on *lama* expression by whole mount *in situ* hybridization. In these mutants, *lama* mRNA is correctly distributed in the third instar brain and in the embryo (data not shown). Qualitatively, the levels of *lama* appear a bit lower. This suggests that both a cryptic transcription start site, and many of the gene's regulatory sequences, lie within its introns. The $lama^{410}$ excision allele is a large, 14 kB deletion, which completely eliminates *lama's* expression.

Phenotypic analysis of lama

We investigated whether animals homozygous for the *lama* null mutation, $lama^{410}$, show developmental abnormalities within the visual system. Proliferation in the third instar brain was detected with an *in vitro* pulse of BrdU. Retinal innervation was analyzed using a photoreceptor specific antibody, MAb24B10 (Zipursky, et. al., 1986). The neuronal and glial cell body layers were visualized with antibodies to neuronal and glial cell type specific markers. The nuclear neuronal antigen, ELAV (Rabinow et. al., 1988) was detected by the MAb44C11 antibody (Bier, et. al., 1988). The nuclear glial

antigen, RK2, was detected by the RK2-5' antisera (Campbell, et. al., 1994). In the absence of *lama* function, the lamina appears to develop normally with respect to cell proliferation (n=19; Fig. 10A,B), retinal innervation of the brain (n=19; Fig. 10 C,D), and the development of neuronal (n=4; Fig. 10 E,F) and glial (n=20; Fig. 10 G,H) cell body layers.

The structures of the adult lamina and retina were also examined in $lama^{410}$ mutants. An analysis of frozen sections indicate that the ganglia are correctly organized and correctly express the neuronal marker, ELAV (n=10; data not shown). Higher resolution was obtained by examining the structure of the visual system in plastic sections (Fig. 11 A,B). No obvious structural abnormalities were observed in the mutants (n=6).

We investigated whether disrupting the spatial organization of *lama's* expression perturbs lamina development. The *lama* cDNA was placed under control of the hsp-70 promoter. Ectopic expression of *lama* during larval life is not lethal, and does not appear to affect the development of the lamina. Following multiple heat pulses, the brains of animals carrying the *hs-lama* transgene were analyzed for cell proliferation (n=7), axon placement (n=18), neuronal development (n=24), and glial development (n=11). As in the null mutant, all of these processes appear to occur normally. Adult animals are fertile, and the structure of the adult visual system resembles that seen in wild type animals (n=1) (data not shown).

It is possible that *lama* mutations cause a subtle, as yet undetected, developmental disruption. Although *lama* mutants behave normally in a crude assay for visual behavior (Perez and Steller, unpublished observations), it is possible that a more sophisticated analysis of visual behavior would indicate aberrant vision in the mutants. Alternatively, *lama* may be a member of a family of proteins which perform the same functions. Low stringency Southern analysis has indicated that the *lama* ORF detects related sequences in *D. melanogaster* (data not shown), which is consistent with *lama* encoding a redundant function.

Isolation and characterization of a *D. virilis lama* homologue

lama mutants show no obvious phenotype, and the sequence of *lama* indicates that it encodes a pioneer protein. In order to (1) determine whether *lama* is likely to encode an important function and (2) identify regions

within *lama* that are likely to be required for its function, we looked for a *D. virilis* homologue of *lama*. *D. virilis* and *D. melanogaster* are diverged by 60 million years (Beverley and Wilson, 1984). Comparisons between *D. melanogaster* and *D. virilis* clones have previously identified regions of functional importance in proteins, and have also indicated conserved regulatory sequences (Heberlein and Rubin, 1990; Yao and White, 1991).

A portion of the *lama* ORF (residues 56-542) was used to screen a genomic *D. virilis* library. One clone was obtained, and the homology was localized to a 6.6 kB R1 fragment. Partial sequence was obtained from the *D. virilis* clone. The *D. virilis* lama protein is 74% identical to the *D. melanogaster* protein over its entire length (Fig. 12). This level of conservation is about average for genes which are essential for visual system development (Heberlein and Rubin, 1990; Michael et. al., 1990; Neufeld et. al., 1990; Hart et. al., 1993). *lama* is more conserved than either *rough* or *sevenless*, but not as well conserved as *bride-of-sevenless* or *seven-in-absentia*.

There are two gaps within the region of overlap. The homology between the clones is disrupted at the 5' end of the *D. virilis* clone by a consensus splice acceptor site (Darnell et. al., 1986). The splice site lies between the two, in frame methionines (italics) found in the *D. melanogaster* clone. The second methionine is not conserved in the *D. virilis* clone, which codes for an isoleucine at that position (asterisk). Partial sequence of the *D. virilis* motif 1 (residues 54-82 within the *D. melanogaster* clone) indicates that it is 89% identical to the corresponding region in the *D. melanogaster* protein. Motif 2 appears to be 72% identical between the proteins.

The expression of *D. virilis lama* was examined in *D. virilis* larval brains via whole mount *in situ* hybridization. The clone appears to be expressed in the *D. virilis* brain (Fig. 13B) in the same pattern that the *D. melanogaster* clone is expressed in the *D. melanogaster* brain (Fig. 13A). At this stage, the proliferation pattern in *D. virilis* brains also resembles that seen in late third instar *D. melanogaster* brains (data not shown).

Taken together, these data suggest that *lama* encodes an evolutionarily conserved protein which functions in the developing optic ganglion.

DISCUSSION

A number of loci which coordinate optic lobe development in *Drosophila* have been identified. The *(l)1trol* and *ana* loci control the proliferation of the optic lobe neuroblasts (Datta and Kankel, 1992; Ebens, et. al., 1993; Datta, 1995). *l(1)optomotor blind* is required to establish the correct structure of the optic lobes (Pflugfelder et. al., 1992). Both *wingless* (*wg*) and *decapentaplegic* (*dpp*) are necessary for the establishment of dorsal/ventral polarity in the optic lobe, and for the differentiation of medulla neurons (Kaphingst and Kunes, 1994). However, few genes which control the development of the first optic ganglion, the lamina, have been identified.

The formation of the lamina is particularly interesting because it is the target field for the majority of the *Drosophila* photoreceptors. Lamina development is intricately linked to the development of the eye. Retinal innervation of the brain induces the proliferation of the lamina neuronal precursors. One locus, *division abnormally delayed* (*dally*), is required for the correct timing of the cell cycle in the lamina's neuronal precursors (Nakato et. al., 1995). Axonal cues are also required for the terminal differentiation of the lamina glia, as well as for the completion of glial migration (Winberg et. al., 1992; Perez and Steller, submitted).

We have isolated a novel gene, *lama*, (*lamina ancestor*) which is expressed in both the lamina's glial and neuronal precursors. The onset of *lama's* expression in the brain is completely independent of retinal innervation. The L-glial precursors begin to express *lama* two days before the onset of retinal innervation, while expression in the L-neuron precursors is coincident with input from the eye. Once the precursors have been incorporated into the developing ganglion, *lama* is down-regulated. The timing of *lama's* expression in the precursors suggests that the locus has a role in the communication between the ingrowing axons and their target field. Alternatively, the gene may be involved in specification of a "lamina" identity.

In animals homozygous for a null mutation in *lama*, the visual system appears to develop normally. Furthermore, ectopic expression of the gene fails to cause any obvious developmental abnormalities. Despite the phenotypic data, we believe that the gene plays a role in the development of the visual system. *lama* encodes a novel protein, which is 74% identical to its

D. virilis homologue. Furthermore, both genes are expressed in the precursors of the first optic ganglion. This cross species conservation indicates that the locus is under selective pressure, and implies that *lama's* function is important to the organism. Other loci required for normal development are not as well conserved (see Curtis et. al., 1995; O'Neil and Belote, 1992). One explanation for the lack of an observed phenotype is that we have not examined the mutants at a high enough level of resolution to detect abnormalities. *lama* may be required for some subtle aspect of visual system development. A defect in *lama* mutants may only manifest themselves in a harsher environment, or in the event that the animals are subjected to a careful examination of their visual behavior. On the other hand, *lama* may encode a protein with a redundant function. Two motifs within the lama protein appear to be conserved in several *C. elegans* genes, suggesting that lama may be a member of a gene family.

Several examples of partial, or complete, genetic redundancy have been described in the literature (reviewed in Thomas, 1993). In some cases, the phenotype of a null mutation does not affect the processes its expression pattern would suggest. Deletion of the *Drosophila brain specific homeobox* locus has no obvious effect on brain morphology, but may be lethal to the organism (Jones and McGinnis, 1993). A *C. elegans* gene, lin-10, is widely expressed but appears to function only in vulval cell fate determination (Kim and Horvitz, 1990). In other cases, null mutations have no apparent phenotype. Vulval development in *C. elegans* requires genes from two different pathways, and animals with mutations in only one of the pathways are wild type (Ferguson and Horvitz, 1989). Loss of one of the major *Drosophila* microtubule associated proteins, 205K MAP, does not appear to affect the development, viability, or fertility of the organism (Pereira, et. al., 1992).

A function for *lama* may be identified by isolating mutations in a closely related gene, and examining the double mutants. The *lama* ORF detects additional sequences on genomic Southerns probed at low stringency (Perez and Steller, unpublished observations). If other *lama* family members are identified, mutations in these loci may be isolated by molecularly screening for P element insertions within these genes (Dalby, et. al., 1995).

Alternatively, the effects of a null mutation in *lama* may be examined in a

sensitized genetic background. The phenotype of another non-essential *Drosophila* locus, *zeste*, is evident in conjunction with certain alleles of the *Ultrabithorax* (*Ubx*) locus (Kaufman et. al., 1973; Goldberg et. al., 1989). The product of the *zeste* locus is required for some aspects of *Ubx* transcription (Laney and Biggin, 1992). A number of genes are expressed in a pattern that overlaps *lama's* expression, and would be potential candidates for a double mutant analysis (see. for e.g.., Gonzalez, et. al., 1989; Nguyen, et. al., 1994; Finelli, et. al., 1995).

In the absence of a described function for *lama*, its expression pattern may be used as the basis of a genetic screen. *lama* is expressed in the lamina precursors without cues from the eye, and is down regulated upon retinal innervation. Mutations which disrupt the early development of the neuronal or glial precursors, or which interrupt retinal signaling to either class of precursors, could be detected.

Within the embryo, several neuroblasts and their decendants express lineage specific markers (see for e.g., Doe et. al., 1988; Patel et. al., 1989). *lama* is unique in two respects. The gene marks the progenitors of specific ganglion, rather than cells of a particular lineage. Furthermore, *lama* marks a distinct developmental stage. *lama* is expressed by the lamina precursors as they are waiting to receive cues from ingrowing retinal axons.

REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* **215,** 403-410.

Ashburner, M. (1989). *Drosophila: a laboratory handbook.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Bairoch, A. (1992). PROSITE: A dictionary of sites and patterns in proteins. *Nucleic Acids Research.* **20,** 2013-2018.

Bairoch, A. and Boeckmann, B. (1992). The SWISS-PROT protein sequence data bank. *Nucleic Acids Research.* **20,** 2019-2022.

Bellen, H.J., O'Kane, C. J., Wilson, C., Grossniklaus, U., Pearson, R. K., and Gehring, W. J. (1989). P element mediated enhancer detection: a versatile method to study development in *Drosophila*. *Genes and Dev.* **3,** 1288-1300.

Beverley, S. M., and Wilson, A. C. (1984). Molecular evolution in *Drosophila* and the higher diptera II: a time scale of evolution. *J. Mol Evol.* **21,** 1-13.

Bier, E., Ackerman, L., Barbel, S., Jan, L.Y., and Jan, Y.N. (1988). Identification and characterization of a neuron specific nuclear antigen in *Drosophila*. *Science.* **240,** 913-916.

Bier, E., Vaessin, H, Shepherd, S., Lee, K., McCall, K., Barbel, S., Ackerman, L., Carretto, R., Uemura, T., Grell, E., Jan, L. Y., and Jan, Y. N. (1989). Searching for pattern and mutation in the *Drosophila* genome with a P-lacZ vector. *Genes and Dev.* **3,** 1273-1287.

Birnstiel, M. L., Busslinger, M., and Strub, K. (1985). Transcription termination and 3' processing: the end is in site. *Cell.* **41,** 349-359.

Bonini, N. M., Leiserson, W. M., and Benzer, S. (1993). The *eyes absent* gene: genetic control of cell survival and differentiation in the developing *Drosophila* eye. *Cell.* **72,** 379-395.

Campbell, G., Göring, H., Lin, T., Spana, E., Andersson, S., Doe, C.Q., and Tomlinson, A. (1994). RK2, a glial specific homeodomain protein required for embryonic nerve cord condensation and viability in *Drosophila*. *Development.* **120,** 2957-2966.

Campos, A. R., Fischbach, K-F., and Steller, H. (1992). Survival of photoreceptor neurons in the compound eye of *Drosophila* depends on connections with the optic ganglia. *Development.* **114,** 355-366.

Campos-Ortega, J. A., and Hartenstein, V. (1985). *The Embryonic Development of Drosophila melanogaster.* Berlin: Springer-Verlag.

Cavener, D. R. (1987). Comparisons of the consensus sequence flanking translational start sites in *Drosophila* and vertebrates. *Nucleic Acids Res.* **15,** 1353-1361.

Cheyette, B.N.R., Green, P.J., Martin, K., Garren, H., Hartenstein, V. and Zipursky, S.L. (1994). The *Drosophila sine oculis* locus encodes a homeodomain containing protein required for the development of the entire visual system. *Neuron.* **12,** 977-996.

Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochem.* **18,** 5294-5299.

Creighton, T. E. (1984). *Proteins: structures and molecular properties.* New York: W.H. Freeman and Company.

Curtis, D., Apfeld, J. and Lehmann, R. (1995). *nanos* is an evolutionarily conserved organizer of anterior-posterior polarity. *Dev.* **121,** 1899-1910.

Dalby, B., Pereira, A. J., and Goldstein, L. S. (1995). An inverse PCR screen for the detection of P element insertions in cloned genomic intervals in *Drosophila melanogaster. Genetics.* **139,** 757-766.

Darnell, J., Lodish, H., and Baltimore, D. (1986). *Molecular Cell Biology.* New York: Scientific American Books, Inc.

Datta, S. (1995). Control of proliferation in quiescent neuroblasts of the *Drosophila* central nervous system. *Development.* **121,** 1173-1182.

Datta, S. and Kankel, D. R. (1992). *l(1) trol* and *l(1) devl,* loci affecting the development of the adult central nervous system in Drosophila melanogaster. *Genetics.* **130,**523-537.

Doe, C. Q., Hiromi, Y., Gehring, W. J. and Goodman, C. S. (1988). Expression and function of the segmentation gene *fushi tarazu* during *Drosophila* neurogenesis. *Science.* **239,** 170-175.

Ebens, A.J., Garren, H., Cheyette, B. N. R., and Zipursky, S. L. (1993). The Drosophila *anachronism* locus: a glycoprotein secreted by glia inhibits neuroblast proliferation. *Cell.* **74**,15-27.

Ephrussi, A., Dickinson, L. K., and Lehmann, R. (1991). *Oskar* organizes the germ plasm and directs localization of the posterior determinant *nanos*. *Cell.* **66(1)**, 37-50.

Ferguson, E. L., and Horvitz, H. R. (1989). The multivulval phenotype of certain *Caenorhabditis elegans* mutants results from defects in two functionally redundant pathways. *Genetics.* **123 (1)**, 109-121.

Finelli, A. L., Xie, T., Bossie, C. A., Blackman, R. K., and Padgett, R. W. (1995). The *tolkein* gene is a *tolloid*/BMP-1 homologue that is essential for *Drosophila* development. *Genetics.* **141**, 271-281.

Fischbach, K.-F., and Technau, G. (1984). Cell degeneration in the developing optic lobes of the *sine oculis* and *small optic lobes* mutants of *Drosophila melanogaster*. *Dev. Biol.* **104**, 219-239.

Goldberg, M. L., Colvin, R. A., and Mellon, A. F. (1989). The *Drosophila zeste* locus is nonessential. *Genetics.* **123**, 145-155.

Gonzalez, F., Romani, S., Cubas, P., Moldolell, J., and Campuzano, S. (1989). Molecular analysis of the *asense* gene, a member of the *achaete-scute* complex of *Drosophila melanogaster*, and its novel role in optic lobe development. *EMBO J.* **8(12)**, 3553-3562.

Grether, M. E., Abrams, J. A., Agapite, J. A., White, K., and Steller, H. (1995). The head involution defective gene of *Drosophila melanogaster* functions in programmed cell death. *Genes and Dev.* **9**,1694-1708.

Hafen, E., and Basler, K. (1991). Specification of cell fate in the developing *Drosophila* eye. *Development.* **1**,123-120.

Hart, A. C., Harrison, S. D., Van Vactor, D. L., Rubin, G. M. and Zipursky, S. L. (1993). The interaction of *bride of sevenless* with *sevenless* is conserved between *Drosophila virilis* and *Drosophila melanogaster*. *PNAS.* **90**, 5047-5051.

Heberlein, U., and Moses, K. (1995). Mechanisms of *Drosophila* retinal morphogenesis: the virtues of being progressive. *Cell.* **81(7)**, 987-990.

Heberlein, U., and Rubin, G. M. (1990). Structural and functional comparisons of the *Drosophila virilis* and *Drosophila melanogaster rough* genes. *PNAS.* **87(15),** 5916-5920.

Henikoff, S. and Henikoff, J. G. (1994). Protein family classification based on searching a database of blocks. *Genomics.* **19,** 97-107.

Hofbauer, A. and Campos-Ortega, J. A. (1990). Proliferation pattern and early differentiation of the optic lobes in *Drosophila melanogaster. Roux's Arch. Devl. Biol.* **149,** 134-148.

Jan, L.Y. and Jan, Y.N. (1982). Antibodies to horseradish peroxidase as specific neuronal markers in *Drosophila* and in grasshopper embryos. *Proc. Natl. Acad. Sci. USA.* **79,** 2700-2704.

Jones, B., and McGinnis, W. (1993). A new *Drosophila* homeobox gene, *bsh,* is expressed in a subset of brain cells during embryogenesis. *Development.* **117,** 793-806.

Kaphingst, K. and Kunes, S. (1994). Pattern formation in the visual centers of the *Drosophila* brain: *wingless* acts via *decapentaplegic* to specify the dorsoventral axis. *Cell.* **78,** 437-448.

Kaufman, T. C., Tasaka, S. E., and Suzuki, D. T. (1973). The interaction of two complex loci, *zeste* and *bithorax* in *Drosophila melanogaster. Genetics.* **75,** 299-321.

Kim, S. K., and Horvitz, H. R. (1990). The *Caenorhabditis elegans* gene lin-10 is broadly expressed while required specifically for the determination of vulval cell fates. *Genes and Dev.* **4(3),** 357-371.

Klämbt, C. (1993). The *Drosophila* gene *pointed* encodes two ets like proteins which are involved in the development of the midline glia. *Development.* **117,** 163-176.

Laney, J. D. and Biggin, M. D. (1992). *zeste,* a nonessential gene, potently activates *Ultrabithorax* transcription in the *Drosophila* embryo. *Genes and Dev.* **6,** 1531-1541.

Laski, F. A., Rio, D. C., and Rubin, G. M. (1986). Tissue specificity of *Drosophila* P element transposition is regulated at the level of mRNA splicing. *Cell.* **44,** 7-19.

Martin, K. A., Poeck, B., Roth, H., Ebens, A. J., Ballard, L. C. and Zipursky, S. L. (1995). Mutations disrupting neuronal connectivity in the *Drosophila* visual system. *Neuron.* **14,** 229-240.

Meinertzhagen, I. A. (1973). Development of the compound eye and optic lobe of insects. In *Developmental Neurobiology of Arthropods.* (ed. D. Young). pp 51-104. Cambridge: Cambridge University Press.

Meinertzhagen, I.A. and Hanson, T.E. (1993). The development of the optic lobe. In *The Development of Drosophila melanogaster,* (eds M. Bate and A. Martinez-Arias). pp 1363-1491. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Meyerowitz, E. M., and Kankel, D. R. (1978). A genetic analysis of visual system development in *Drosophila melanogaster. Dev. Biol.* **62,** 112-142.

Michael, W., Bowtell, D. and Rubin, G. M. (1990). Comparison of the *sevenless* genes of *Drosophila virilis* and *Drosophila melanogaster. PNAS.* **87,** 5351-5353.

Mlodzik, M. and Hiromi, Y. (1992). The enhancer trap method in Drosophila: its application to neurobiology. In *Methods in Neuroscience, vol. 9: Gene Expression in Neural Tissues.* (ed. P. M. Cann) pp. 397-414. New York:Academic Press.

Nakato, H., Futch, T. A. and Selleck, S. B. (1995). The *division abnormally delayed* (*dally*) gene; a putative integral membrane protein required for cell division patterning during post-embryonic development in *Drosophila. Dev.* **121,** 3687-3702.

Neufeld, T. P., Carthew, R. W. and Rubin, G. M. (1991). Evolution of gene position: chromosomal arrangement and sequence comparison of the *Drosophila melanogaster* and *Drosophila virilis sina* and *Rh4* genes. *PNAS.* **88,** 10203-10207.

O'Neil, M. T. and Belote, J. M. (1992). Interspecific comparison of the *transformer* gene of *Drosophila* reveals an unusually high degree of evolutionary divergence. *Genetics.* **131,** 113-128.

Pardue, M.L. (1994). Looking at polytene chromosomes. In *Drosophila melanogaster: practical uses in cell and molecular biology.* (eds. L. S. B. Goldstein and E. A. Fyrberg) pp. 219-239. New York: Academic Press.

Patel, N. H., Schafer, B., Goodman, C. S. and Holmgren, R. (1989). The role of segment polarity genes during *Drosophila* neurogenesis. *Genes and Dev.* **3,** 890-904.

Pearson, W. R. (1990). Rapid and sensitive sequence comparison with FASTP and FASTA. *Methods in Enzymology.* **183,** 63-98.

Pereira, A., Doshen, J., Tanaka, E., and Goldstein, L. S. B. (1992). Genetic analysis of a *Drosophila* microtubule-associated protein. *JCB.* **116(2),** 377-383.

Perez, S. E. and Steller, H. Migration of glial cells into the retinal target field in *Drosophila melanogaster, submited.*

Pflugfelder, G. O., Roth, H., Poeck, B., Sigl, A., Kerscher, S., Jonschker, B., and Heisenberg, M. (1992). The *l(1)optomotor-blind* gene of *Drosophila melanogaster* is a major organizer of optic lobe development: isolation and characterization of the gene. *PNAS.* **89,** 1199-1203.

Pongor, S., Hatsagi, Z., Degtyarenko, K., Fabien, P., Skerl, V., Hegyi, H., Murvai, J., and Bevilacqua, V. (1995). The SBASE protein domain library releas 3.0: a collection of annotated protein sequence segments. *Nucleic Acids Research.* **22,** 3610-3615.

Power, M. E. (1943). The effect of reduction in numbers of ommatidia upon the brain of *Drosophila melanogaster. J. Exp. Zool.* **94,** 33-71.

Ready, D. F., Hanson, T. E., and Benzer, S. (1976). Development of the *Drosophila* retina, a neurocrystalline lattice. *Dev. Biol.* **53,** 217-240.

Robertson, H. M., Preston, C. R., Phillis, R. W., Johnson-Schlitz, D. M., Denz, W. K., and Engels W. R. (1988). A stable genomic source of P element transposase in *Drosophila melanogaster. Genetics.* **118,** 461-470.

Robinow, S., Campos, A.R., Yao, K.M., White, K. (1988). The *elav* gene product of *Drosophila,* required in neurons, has three RNP consensus motifs. *Science.* **242,** 1570-1572.

Saiki, R. K. (1990). Amplification of genomic DNA. In *PCR Protocols.* (eds. M. A. Innis, D. H. Gelfand, J. J. Sinsky, and T. J. White). pp. 13-20. New York: Academic Press.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). *Molecular cloning: a laboratory manual.* Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York.

Schneider, T., Reiter, C., Eule, E., Bader, B., Lichte, B., Nie, Z., Schimansky, T., Ramos, R. G. P. and Fischbach, K. F. (1995). Restricted expression of the *irreC-rst* protein is required for normal axonal projections of columnar visual neurons. *Neuron.* **15,** 259-271.

Selleck, S.B. and Steller, H. (1991). The influence of retinal innervation on neurogenesis in the first optic ganglion of *Drosophila. Neuron.* **6,** 83-99.

Smith, D., Wohlgeruth, J., Calvi, B. R., Franklin, I., and Gelbart, W. M. (1993). hobo enhancer trapping mutagenesis in *Drosophila* reveals an insertional specificity different from P elements. *Genetics.* **135(4),** 1063-1076.

Spurr, A. R. (1969). A low viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastructur Res.* **26,** 31-43.

Steller, H., and Pirotta, V. (1986). P transposons controlled by the heat shock promoter. *Mol. Cell Biol.* **6,** 1640-1649.

Stewart, R. J., Pesavento, P.A., Woerpel, D.N., and Goldstein, L. S. B. (1991). Identification and partial characterization of six members of the kinesin superfamily in Drosophila. *PNAS.* **88,** 8470-8474.

Tautz, D. and Pfeifle, C. (1989). A non-radioactive in situ hybridization method for the localization of specific RNAs in Drosophila embryos reveals translational control of the segmentation gene hunchback. *Chromosoma.* **98,** 81-85.

Thomas, J. H. (1993). Thinking about genetic redundancy. *Trends in Genetics.* **9(11),** 395-400.

Waterston, R., Martin, C., Craxton, M., Huynh, C., Coulson, A., Hillier, L., Durbin, R., Green, P., Shownkeen, R., Halloran, N., Metzstein, M., Hawkins, T., Wilson, R., Berks, M., Du, Z., Thomas, K., Thierry-Mieg, J., and Sulston, J. (1992). A survey of expressed genes in *Caenorhabiditis elegans. Nature Genetics.* **1,** 114-123.

White, K. and Kankel, D.R. (1978). Patterns of cell division and cell movement in the formation of the imaginal nervous system of *Drosophila melanogaster. Dev. Biol.* **104,** 219-239.

Wilson, C., Pearson, R. K., Bellen, H. J., O'Kane, C. J., Grossniklaus, U., and Gehring, W. (1989). P element mediated enhancer detection: isolation and characterization of developmentally regulated genes. *Genes and Dev.* **3,** 1301-1333.

Wilson, C., Goberdahn, D. C. I., and Steller, H. (1993). *Dror,* a potential neurotrophic receptor gene encodes a *Drosophila* homolog of the vertebrate Ror family of Trk-related receptor tyrosine kinases. *PNAS.* **90,** 7109-7113.

Winberg, M.L., Perez, S.E., and Steller, H. (1992). Generation and early differentiation of glial cells in the first optic ganglion of *Drosophila melanogaster. Development.* **115,** 903-911.

Wolff, T. and Ready, D. (1991). The beginning of pattern formation in the *Drosophila* compound eye: the morphogenetic furrow and the second mitotic wave. *Development.* **113(3),** 841-850.

Worley, K. C., Wiese, B. A., and Smith, R. F. (1995). BEAUTY: An enhanced BLAST-based search tool that integrates multiple biological information resources into sequence similarity search results. *Genome Research.* **5,** 173-184.

Xiong, W-C. and Montell, C. (1995). Defective glia induce neuronal apoptosis in the *repo* visual system of *Drosophila*. *Neuron.* **14,** 581-590.

Yao, K. M., and White, K. (1991). Organizational analysis of *elav* gene and functional analysis of elav protein of *Drosophila melanogaster* and *Drosophila virilis*. *Mol. Cell Biol.* **11(6),** 2994-3000.

Zak, N.B. and Shilo, B-Z. (1992). Localization of DER and the pattern of cell divisions in wild type and ellipse eye imaginal discs. *Dev. Biol.* **149,** 448-456.

Zinn, K., McAllister, L., and Goodman, C.S. (1988). Sequence analysis and neuronal expression of fasciclin I in grasshopper and *Drosophila*. *Cell.* **53(4),** 577-587.

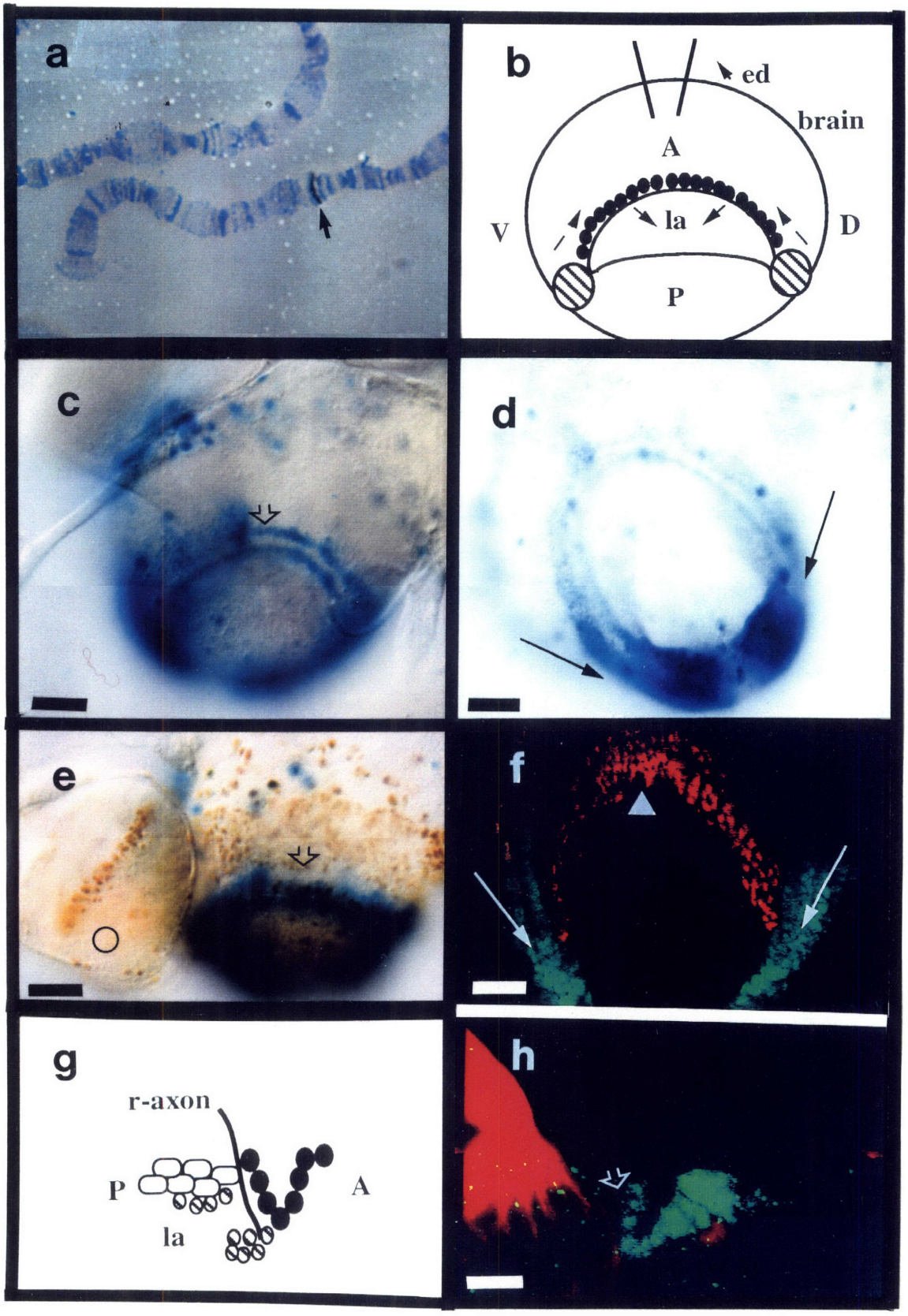
Zipursky, S. L., and Rubin, G. M. (1994). Determination of neuronal cell fate: lessons from the R7 neuron of *Drosophila*. *Ann. Rev. Neuroscience.* **17,** 373-397.

Zipursky, S., Venkatesh, T., and Benzer, S. (1986). From monoclonal antibody to gene for a neuron-specific glycoprotein in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **82,** 1855-1859.

Acknowledgments: We thank W. Gelbart, for providing the Hop6yw chromosome, J. Tamkun, for providing the *D. virilis* library and to M. Chafel, for plastic sectioning. We are grateful to L. Goldstein for sharing data prior to publication. We thank K. Mejia for assistance in screening the *D. virilis* library, F. Davidson for assistance in embedding samples for histology, and K. Roberg for assistance with database searches. We are grateful to M. Grether, K. Roberg and J. Kim for critical comments on the manuscript.

Figure 1: Late third instar expression of a lamina precursor marker.

(A) Cytological location of P element lines which expresses the β–galactosidase reporter in cells surrounding the lamina. A polytene squash from the PZA5 line was probed with pBluescript. The P insertions map to 64C11 (arrow). In C-F, anterior is to the top of the page. (B) Schematic of the late third instar lamina, viewed laterally. The brain, lamina (la), and location of the eye disc (ed) are indicated. The anterior/posterior (A,P) and dorsoventral (D,V) axes are also indicated. Lamina neuronal (L-neurons) precursors are shown as filled circles, and are found at the tissue's anterior border. The lamina glial (L-glia) precursor zones, located at the dorsal and ventral posterior borders of the lamina, are shown as stippled circles. The migration routes of the lamina precursors into the tissue are indicated by arrows. (C) βgalactosidase reporter expression, detected via an X-gal stain, is seen at the anterior border of the lamina (arrowhead). (D) β–galactosidase reporter expression, detected via an X-gal stain, is seen at the dorsal and ventral posterior corners of the lamina (arrows). (E) β–galactosidase reporter expression (blue) overlaps with lamina neurogenesis (brown). The L-neuron precursors are indicated with an arrowhead. The eye disc is indicated with an open circle. Reporter expression is detected via an X-gal stain, and S phases are detected by a BrdU pulse followed by immunohistochemistry. (F) βgalactosidase reporter expression (green) is seen adjacent to the L-glial layers (red). The posterior margin of the lamina is indicated with a triangle. Reporter expression is detected with anti-β–galactosidase, and the L-glia are detected with a glial specific antibody, anti-RK2. In (G,H), anterior is to the right. (G) Schematic of a late third instar lamina, viewed horizontally. A single photoreceptor axon (r-axon) is drawn. L-neuron precursors, shown as filled circles, are distributed along a furrow at the lamina's anterior border. The L-neurons are shown as ovals, and the L-glia are shown as stippled circles. (H) β–galactosidase reporter expression (green) is contiguous around the anterior lamina furrow, which lies adjacent to retinal axons (red). The posterior wall of the furrow is indicated with an arrowhead. Reporter expression is detected with anti-β–galactosidase, R-axons are detected with a general neuronal marker, anti-HRP. Scale bars: C-F, 12.5 μm; H, 33 μm.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b
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Figure 2. Expression of *lama* in the brains of young larvae.

Anterior is to the left in all panels. Expression of *lama* is shown via an X-gal stain (blue), except in (E), where it is detected via anti-β–galactosidase. (A) *lama* expression at approximately 26 hours AEL. Two, symmetrical zones of *lama* expression are indicated by arrows. (B) *lama* expression at 50 hours AEL. The symmetrical zones of *lama* expression are indicated by arrows, and will be referred to henceforth as the "L-glial precursor zones". (C) *lama* expression in a young third instar brain, just prior to retinal input. The L-glial precursor zones are indicated by arrows. The optic stalk is indicated with an open triangle. (D) *lama* expression (blue) in a newly innervated brain. The brain is counter stained with BrdU to detect cell proliferation (brown). The L-glial precursors zones are indicated by arrows, and a row of L-neuron precursors is indicated with an arrowhead. (E) *lama* expression (green) is shown relative to the placement of retinal axons (red). The eye disc is indicated with an open circle, and the optic stalk is marked with an open triangle. A row of L-neuron precursors is indicated with an arrowhead. *lama* expression is detected with anti-βgalactosidase, and the retinal axons are detected with a photoreceptor specific antibody, MAb 24B10. (F) *lama* expression within 8 hours of innervation (approximately 102 hours AEL). The L-glial precursor zones are indicated by arrows, and a cluster of L-neuron precursors is indicated by an arrowhead. The optic stalk is indicated with an open triangle. Scale bars: A, B, E, 5 μm; C, D, F, 12.5 μm

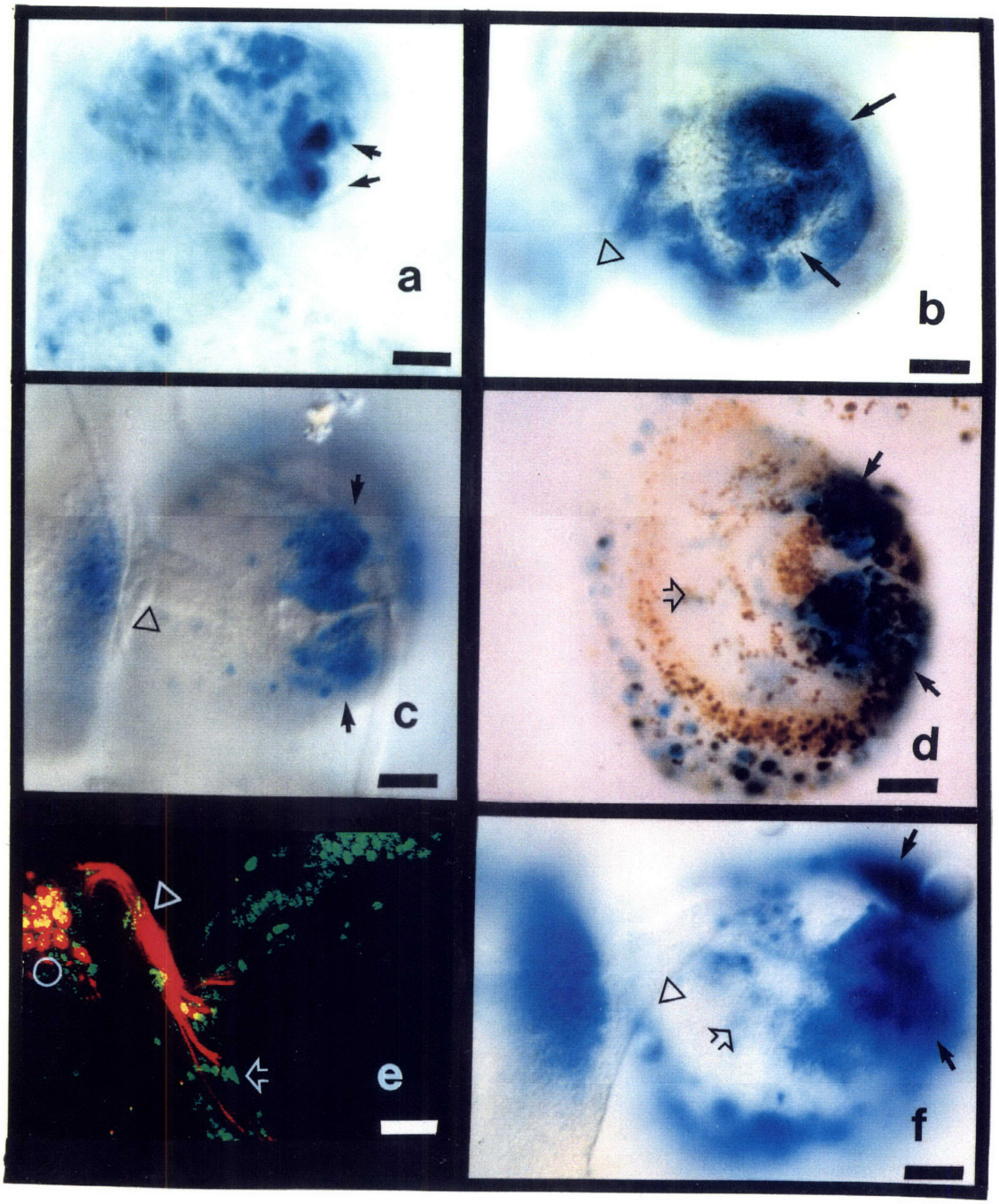
a
b
c
d
e
f

Figure 3. Expression of *lama* in the absence of input from the eye.

Anterior is to the top of the page. *lama* expression is detected via an X-gal stain. Expression is seen in both classes of lamina precursors in the absence of retinal innervation. Expression in the L-neuronal precursors is indicated with an arrowhead, and expression in the L-glial precursor zones is indicated with arrows. (A) *lama* expression in a *sine oculis*^{1} animal. (B) *lama* expression in an *eyes absent* animal. Scale bars: 12.5 μm

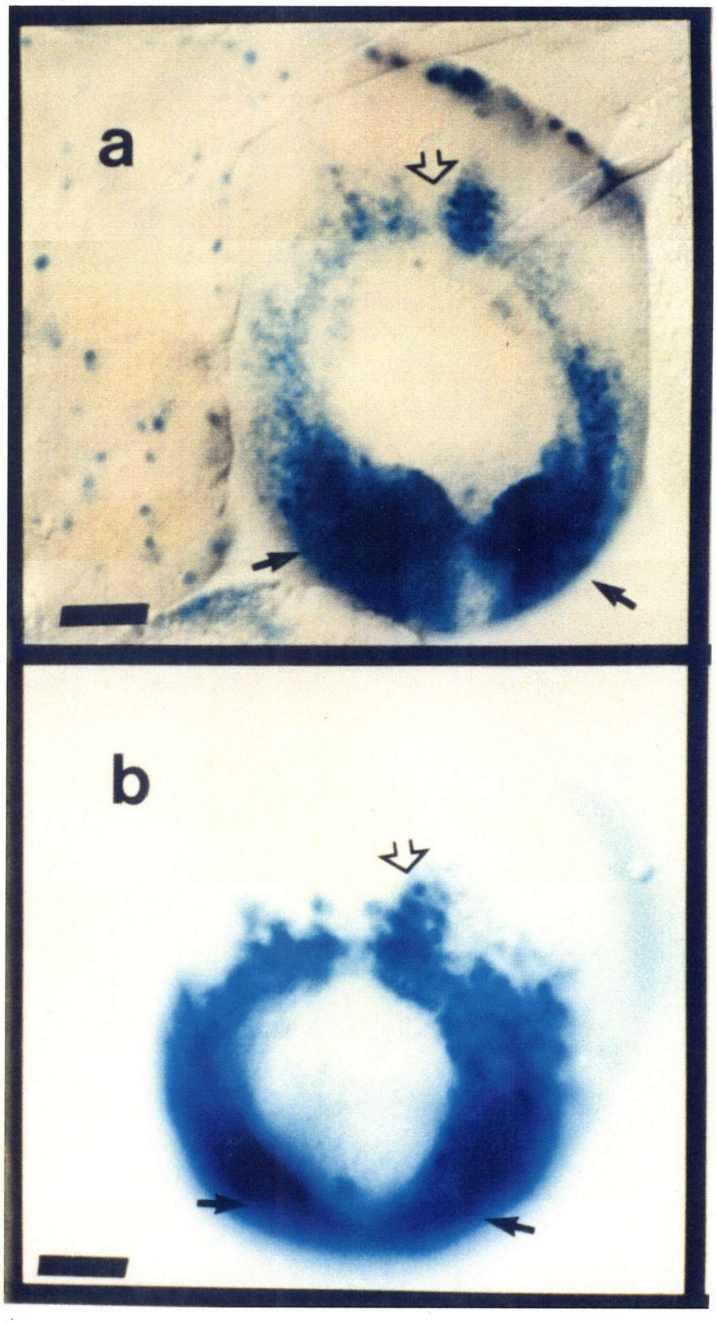
a
b

Figure 4. Genomic region surrounding P element insertions at 64C11.

A genomic map was established by Southern analysis of overlapping lambda clones. Fragments which detect homology on *D. virilis* Southerns are indicated with asterisks. The P element insertions are represented by filled triangles. All P elements are inserted within 1 kB of each other, and are 3' to KLP64D and 5' to *lama*. Arrows indicate the direction of transcription. An additional 3 kB of genomic DNA 3' to *lama* was isolated (not shown). Exons are designated by rectangles, and the coding sequences are indicated by filled rectangles. (R) Ecor1, (C) Cla1, (Sp) Spe1, (H) HindIII, (S) Sac1, (Hp) Hpa1, (E) Eag1, (P) Pst1, (B) Bamh1.

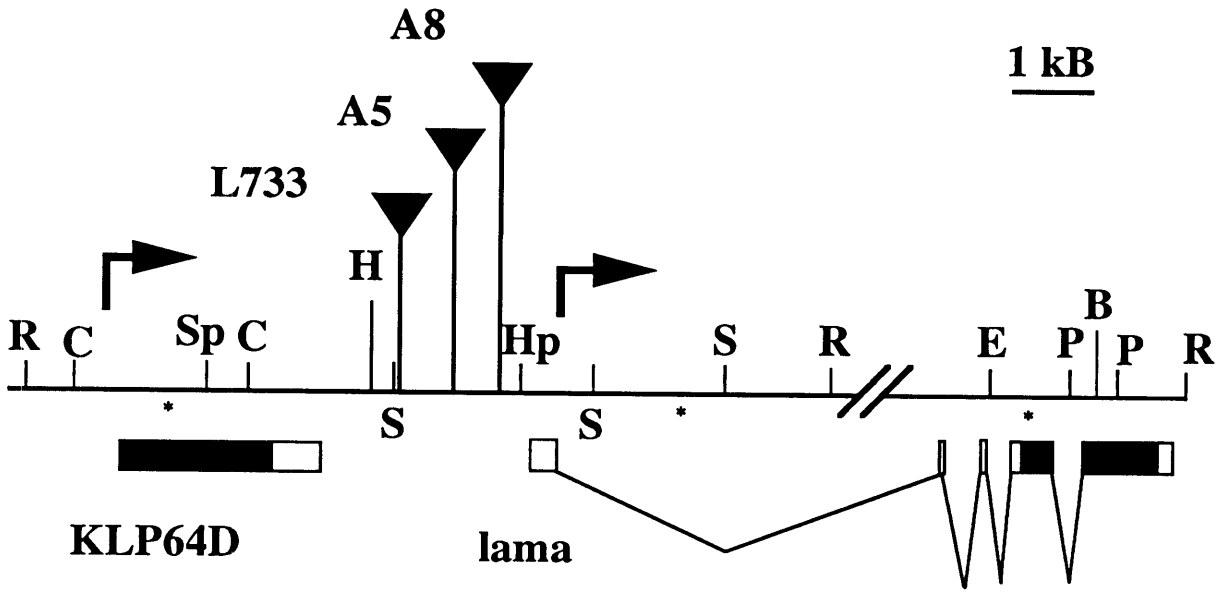
A8
A5
L733
1 kB
H
R
C
Sp
C
Hp
S
R
E
P
B
P
R
S
S
KLP64D
lama

Figure 5. Expression of *lama* mRNA during embryogenesis.

Anterior is to the left in each panel. *lama* expression is shown via whole mount *in situ* hybridization. (A) Between 3 to 3.5 hours AEL, *lama* is seen in the region of the cephalic furrow (arrowhead). (B,C) Between 5 and 7 hours AEL, *lama* is expressed in the ventral nerve cord. Expression is segmentally reiterated, and is seen to either side of the midline (arrow). Expression is also seen in the brain (arrowhead). (D) In older embryos, strong expression is seen in the brain (arrowhead) and in the gonadal progenitors (arrow).

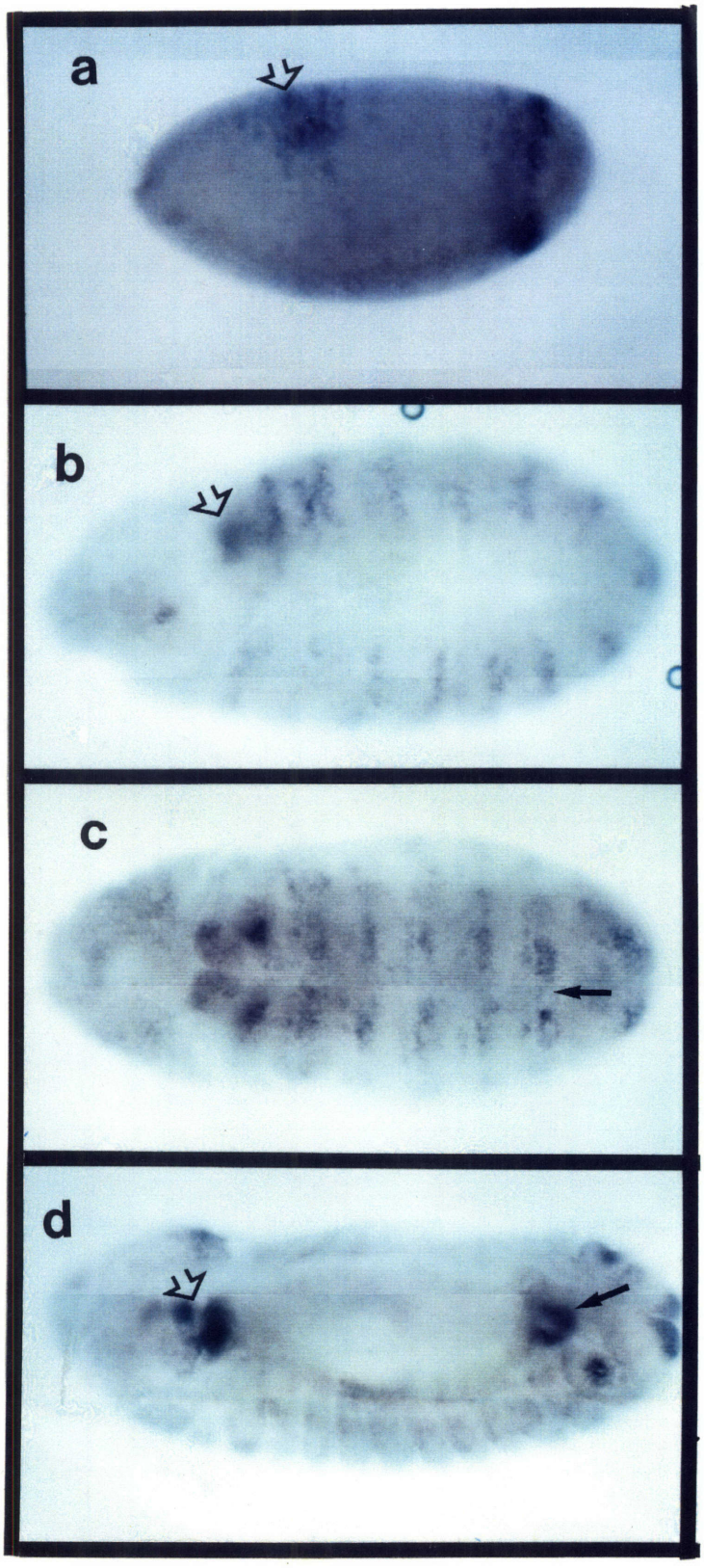
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Figure 6. Post-embryonic expression of *lama* mRNA.

Anterior is to the left in each panel. *lama* expression is shown via whole mount *in situ* hybridization. (A) In late third instar larval brains, expression is seen in the lamina precursors. The progenitors of the lamina neurons are found at the anterior margin of the developing tissue, and are indicated with an arrowhead. The lamina glia arise from distinct regions of the OPC, which are located at the ganglion's dorsal and ventral posterior margins. The glial precursor zones are indicated by arrows. (B) Expression is seen in ovarian follicle cells.

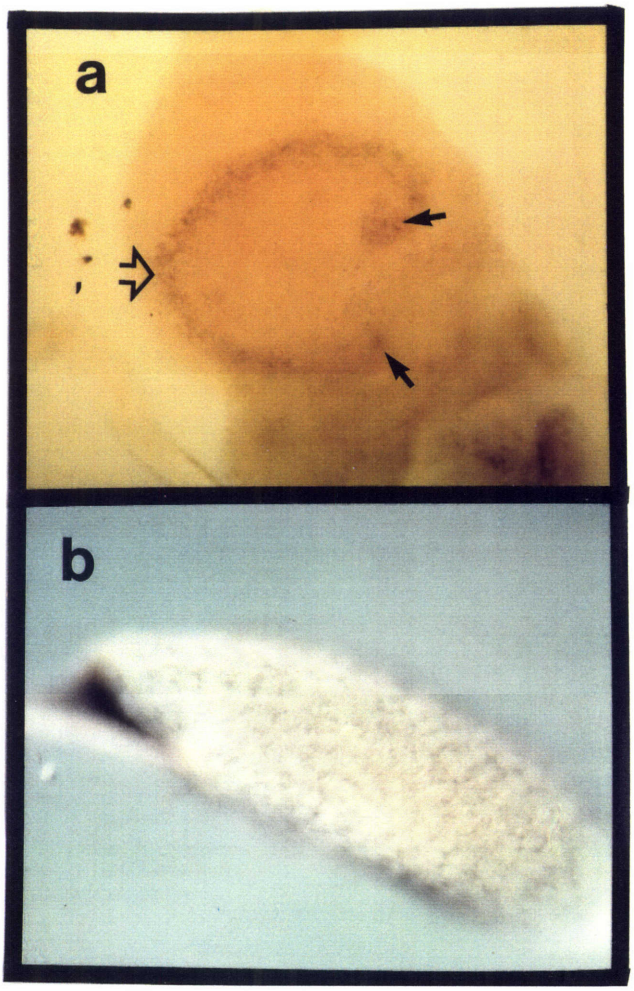
a
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Figure 7. The cDNA and putative protein sequence of *lama*.

The sequence of the lama cDNA is shown above the predicted amino acid sequence of the lama ORF. Numbers to the left indicate base pair positions within the nucleic acid sequence, while numbers to the right indicate amino acid residues within the coding sequence. Two, in frame methionine residues, which fit well with *Drosophila* consensus start sequences, are italicized. Exon-intron boundaries are indicated in bold. The poly-adenylation consensus site is underlined and in bold. Open circles indicate consensus N linked glycosylation sites, and serine residues which are a good fit with the cAMP kinase consensus are underlined.

```
   1  GAATTCCATTAGTTACTAGCCGTTGATATTTTCAATACGCCGCATAGCAACAATAACCAT
  61  ATCAAACATTTCGATAATCTGATGTGACACAAGTTTTTGCATCGAAATAGCCGTGTGTGT
 121  GTGTGTGTGTTTGGTTCCCATTCGCCGCCAATCAAACCAATCGCATTGAGAGTGTAAT
 181  CCGGGAAAAGTTATCACACACCTAAACCTGCGCCTTTTCAATGTAGCCCTTCATGGATAA
 241  TTAACACGTGAGCGTTGTGTGACTATATAGCAAAATCGTAGTTAAAATCGTGTTCGCAGT
 301  GGCTGAATTTAGTAGCATTCCAACGAATAGTGTCATCTCGAACCCAGACACCCCCCCCAC
 361  CCCCTGCGAATTTCTCTTATTTGTCGGCGCGGAATTTGCCTGAGTCACCAGCCAGTTTCA
 421  ATTTTCCGTTCTTAAATGGGTTGACTTGTGATAGTTACTGCTTCCTGGACCCGGGAAAAA
 481  AAAGGGATACAAATTGAAACCTCAAAAGACATAGCCAAGCTGCAATAAAACCATCGCCAA
 541  ACCACCGCCGCCAATTTAAAGTCAATAGTTAGCACGAATATAACCACCTGGAAACCGTTG
 601  GAATCTCTGCTTGAAAGCAGCGCACCACCCATGAACCAATCGGCCAAGGCGCTCAACAGC
 661  GTGGCCACCGAGATCAGCACCTCCTGCTCCTCCAGCACCTACACCAACCATCCACAGAAC
 721  CACCTGCACACTGGCCACCTGATTGCCCACCGTCTTGGACACTCCTACGCCGCTCGTGCC
 781  GCATCACAATTATCTGGGATAAGTTCTGCAATTGGAATCTGACTGGTGAAACACGTTTCT
 841  CGAGAGGATTTTCCATTTTTGCATCGTAGCAGCTTCACGACGCCGGCACATGTAAAAGTC
 901  GTTGGTGCATCGTGGCAAAAAACGCGGATAGGGACCTACATCCTAATCGGTGCTGGTCTC
 961  TTGGTCATCGGAGCCTTCTTCATTGGATATATGGAGAGGCCGGAGTACGATGGCACCTAC
                                   M  E  R  P  E  Y  D  G  T  Y   10
1021  TGTGCCACCGCTCTGTGGACCAAGCAGGTGGGCTTCCAGATTGAAAACTGGAAGCAGCAG
       C  A  T  A  L  W  T  K  Q  V  G  F  Q  I  E  N  W  K  Q  Q   30
1081  AACGATCTGGTCAATATTCCCACGGGTGTTGGCAGGATTTGTTACAAGGATTCGGTTTAT
       N  D  L  V  N  I  P  T  G  V  G  R  I  C  Y  K  D  S  V  Y   50
1141  GAGAATGGTTGGGCCCAAATCGAAGTGGAGACGCAAAGAACTTATCCGGATTGGGTGCAG
       E  N  G  W  A  Q  I  E  V  E  T  Q  R  T  Y  P  D  W  V  Q   70
1201  GCCTATGCCGCCGGAATGCTGGAAGGCTCGCTGACCTGGAGGAACATCTACAATCAGTGG
       A  Y  A  A  G  M  L  E  G  S  L  T  W  R  N  I  Y  N  Q  W   90
1261  TCCAATACCATTTCCTCTTCTTGCGAGCGCGATGAAAGCACCCAGAAATTCTGCGGTTGG
       S  N  T  I  S  S  S  C  E  R  D  E  S  T  Q  K  F  C  G  W  110
1321  CTCAGGGACCTTTTGACCACCAACTACCATCGCCTGAAGAGGCAGACTGAAAAGGCTGAG
       L  R  D  L  L  T  T  N  Y  H  R  L  K  R  Q  T  E  K  A  E  130
1381  AATGATCACTACTGGCATCAGCTGCATCTGTTCATCACCCAGCTGGAGGGCCTGGAAACT
       N  D  H  Y  W  H  Q  L  H  L  F  I  T  Q  L  E  G  L  E  T  150
1441  GGCTACAAGCGAGGAGCATCCCGGGCACGATCCGACTTGGAGGAGGAGATACCATTTTCC
       G  Y  K  R  G  A  S  R  A  R  S  D  L  E  E  E  I  P  F  S  170
1501  GATTTTCTGCTGATGAACGCCGCTGCCGATATTCAGGATCTCAAGATCTATTACGAGAAC
        D  F  L  L  M  N  A  A  A  D  I  Q  D  L  K  I  Y  Y  E  N 190
1561  TATGAGCTGCAGAATAGTACGGAACATACCGAGGAACCCGGAACGGATCAGCCAAAGAAC
       Y  E  L  Q  N°  S  T  E  H  T  E  E  P  G  T  D  Q  P  K  N  210
1621  TTCTTTCTGCCGAGTGCCACCATGCTGACGAAGATCGTCCAGGAGGAGGAGTCGCCGCAG
       F  F  L  P  S  A  T  M  L  T  K  I  V  Q  E  E  E  S  P  Q  230
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       V  L  Q  L  L  F  G  H  S  T  A  G  S  Y  S  S  M  L  R  I  250
1741  CAGAAGCGGTACAAGTTTCACTATCACTTCTCGTCGAAACTGCGGAGTAACACGGTGCCC
       Q  K  R  Y  K  F  H  Y  H  F  S  S  K  L  R  S  N  T  V  P  270
1801  GGTGTGGATATCACTTTCACTGGTTATCCTGGCATTCTCGGATCCACCGATGACTTCTAC
       G  V  D  I  T  F  T  G  Y  P  G  I  L  G  S  T  D  D  F  Y  290
1861  ACCATCAAAGGTCGTCATCTGCATGCAATCGTAGGAGGAGTGGGCATCAAGAACGAGAAT
       T  I  K  G  R  H  L  H  A  I  V  G  G  V  G  I  K  N  E  N  310
1921  CTGCAGCTGTGGAAGACGGTGGATCCGAAGAAGATGGTGCCCTTGGTGGCTCGTGTAATG
       L  Q  L  W  K  T  V  D  P  K  K  M  V  P  L  V  A  R  V  M  330
1981  GCCGCCAATCGGATATCCCAGAATCGTCAGACTTGGGCCAGTGCCATGTCCAGACATCCA
       A  A  N  R  I  S  Q  N  R  Q  T  W  A  S  A  M  S  R  H  P  350
2041  TTTACGGGAGCCAAGCAGTGGATCACCGTTGATCTGAACAAGATGAAGGTGCAGGATAAT
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2101  CTGTACAATGTCCTTGAAGGCGATGACAAGCATGACGATGCTCCAGTAGTGCTGAACGAA
       L  Y  N  V  L  E  G  D  D  K  H  D  D  A  P  V  V  L  N  E  390
2161  AAGGATAGGACAGCCATCCAGCAGAGGCACGATCAGCTAAGGGATATGGTTTGGATTGCC
       K  D  R  T  A  I  Q  Q  R  H  D  Q  L  R  D  M  V  W  I  A  410
2221  GAGCAGCTGCCGGGCATGATGACCAAAAAGGATGTTACTCAGGGATTCCTGGTGCCCGGC
       E  Q  L  P  G  M  M  T  K  K  D  V  T  Q  G  F  L  V  P  G  430
2281  AACACGTCCTGGCTGGCCAATGGAGTGCCGTATTTCAAAAATGTCCTGGAGCTAAGCGGC
       N°  T  S  W  L  A  N  G  V  P  Y  F  K  N  V  L  E  L  S  G  450
2341  GTCAACTACAGCGAGGATCAACAGCTGACGGTGGCCGATGAGGAGGAGCTGACCAGTCTG
       V  N°  Y  S  E  D  Q  Q  L  T  V  A  D  E  E  E  L  T  S  L  470
2401  GCGTCGGTGGATATGTATTTGCGGACTCATGGATTCCGTGGTGATCTGCTGGGCAGCCAG
       A  S  V  D  M  Y  L  R  T  H  G  F  R  G  D  L  L  G  S  Q  490
2461  GAGTCGATTGCCTATGGCAACATAGATCTGAAATTGTTCTCCTACAATGCCCGACTGGGC
       E  S  I  A  Y  G  N  I  D  L  K  L  F  S  Y  N  A  R  L  G  510
2521  ATCAGCGATTTCCATGCCTTTGCGGGTCCAGTCTTTCTGAGATTCCAACATACTCAGCCC
       I  S  D  F  H  A  F  A  G  P  V  F  L  R  F  Q  H  T  Q  P  530
2581  AGGACTCTGGAGGATGAGGGCCAGGATGGTGGTGTTCCACCCGCCGCATCCATGGGCGAC
       R  T  L  E  D  E  G  Q  D  G  G  V  P  P  A  A  S  M  G  D  550
2641  GAACGGCTCAGCGTGAGCATCGAGGATGCAGATAGTCTGGCGGAAATGGAACTCATCACG
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2761  CCCTTCAAGTGGTCAGAGATGAGTCCCGTGGAGGAGGGCGGTGGCCATGAGGGGCATCCT
       P  F  K  W  S  E  M  S  P  V  E  E  G  G  G  H  E  G  H  P  610
2821  GATGAGTGGAACTTTGACAAGGTCTCGCCGAAGTGGGCGTGGTAGGCGCTGAGGAGCAGG
       D  E  W  N  F  D  K  V  S  P  K  W  A  W  *                 624
2881  TTTCTTCTTTTAACCCGTAGAACCCAATCGAATTTGTACCCAAAACCCACCTCCATCCAT
2941  TTTGAAAACTGGAAAGCAATAATTGTCCTAGATTTGGTAATTAGTGTCCTGTCTTGTCTC
3001  GTATAATTTGTGTGTTCCTAGTTGGTACATATCTTACGTCCATAGTCTTCTAGTGTCTAC
3061  TGTACTTATGTATGTATGCGTGTCCAATCGATTATTGTCTTCCAGCTGTACAGCTTTTTA
3121  TTTTCCGTAGGTCTTTTGTAAATTAGCAACGAAATTGGAAATATATTTTTTAGCAACAAA
3181  TTTTTGTACTTTAGTAACTATTAACAAATTGTAAAGAAAATAAAAAACAAAATCATGAAAAAAA
```

Figure 8: Cross species comparison of lama motifs

The lama ORF was compared to sequences in the EMBL, GenBank, PROSITE and SWISS-PROT data bases. The conceptual translations of several *C. elegans* cDNAs suggests that 2 motifs within lama are quite well conserved. Within lama, motif 1 consists of residues 45-82, while motif 2 consists of residues 598-622. Two distinct cDNAs appear to encode motif 1. Within motif 1, cm02a12 is 45% identical to lama, and cm5a8 is 39% identical to lama. An additional cDNA appears to encode motif 2. Within motif 2, yk30d9 is 52% identical to lama. The accession numbers are as follows: Genbank (cm02a12= M75762); EMBL (cm5a8=Z14766); dbj (yk30d9=D33409).

motif 1: **identity**

lama YKDSVYENGWAQIEVETQRTYPDWVQAYAAGMLEGSLT
cm02a12 WQNAVNTTGWTFLEVETKENYCPQLQAYSAGYLEGLLS 45%

lama YKDSVYENGWAQIEVETQRTYPDWVQAYAAGMLEGSLT
cm5a8 FHDEVNQTGWAFLEVDVISPKIPHYLQGYAAGFAEGRAT 39%

motif 2: **identity**

lama SPVEEGGGHEGHPDEWNFDKVSPKW
yk30d9 SPLREKVPHFGQPDRWNFAPVTYKW 52%

Figure 9. Deletion mutations generated by imprecise excision of P elements at 64C11.

The genomic map of the 64C11 region is shown, and the P element insertions are represented by filled triangles. KLP64D and *lama* are represented as rectangles, coding sequences are indicated by filled rectangles, and the direction of transcription is indicated by arrows. Deletion mutations are drawn as filled bars underneath the map.

Five mutations in *lama* were generated by imprecise excision of PZA8. All *lama* mutations are viable and fertile. Deletions into *lama's* first exon are insufficient to disrupt transcript expression. $lama^{410}$ is a null allele which completely deletes the transcript.

Seven mutations in KLP64D were generated, and the P element insertion used to generate each allele is indicated by its name. KLP64D mutations are lethal and do not affect *lama's* expression.

Deletions which retain part of the P elements are indicated with hatched rectangles. (R) Ecor1, (C) Cla1, (Sp) Spe1, (H) HindIII, (S) Sac1, (Hp) Hpa1, (E) Eag1, (P) Pst1, (B) Bamh1.

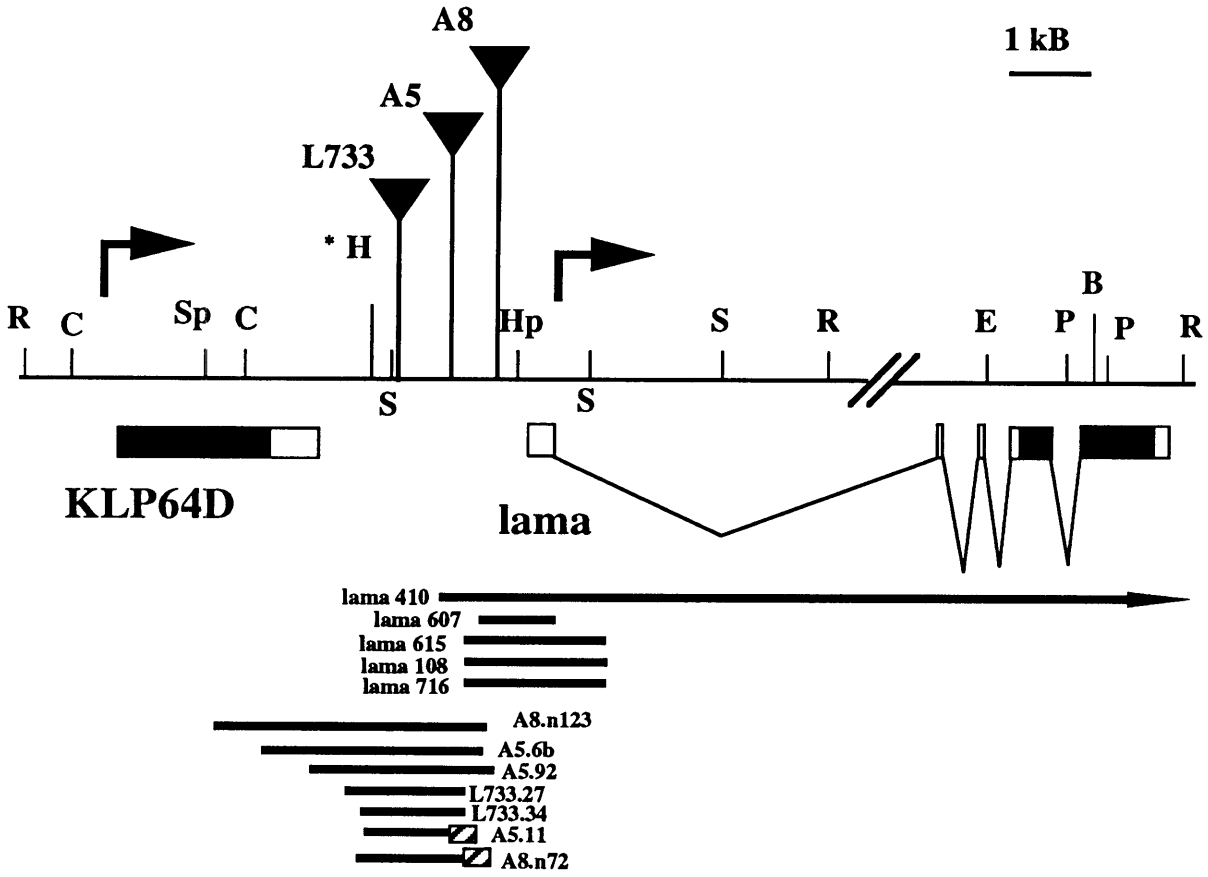

A8
1 kB
A5
L733
* H
B
R
C
Sp
C
Hp
S
R
E
P
P
R
S
S
KLP64D
lama
lama 410
lama 607
lama 615
lama 108
lama 716
A8.n123
A5.6b
A5.92
L733.27
L733.34
A5.11
A8.n72

Figure 10. Analysis of the developing lamina in *lama* mutants.

Anterior is to the left in all panels. (A,C,E,G) show wild type brains, while (B,D,F,H) show animals homozygous for the *lama* ^{410} deletion allele. (A,B) Cell proliferation, detected by a BrdU pulse, appears normal in *lama* mutants. The L-neuron precursors are indicated with arrowheads. (C,D) Retinal innervation of the brain appears normal in *lama* mutants. The photoreceptor axons are detected by MAb24B10. (E,F) The L-neurons appear normal in *lama* mutants. Arrowheads indicate the positions of the lamina cortex. Expression of the neuronal antigen, ELAV, is detected via MAb44C11. (G,H) The L-glial layers appear normal in *lama* mutants. Expression of the glia specific antigen, RK2, is shown in one of the L-glia layers (arrow). Scale bars: A-C, 25 μm; D, H, 17 μm; E,F, 22μm; G, 14μm.

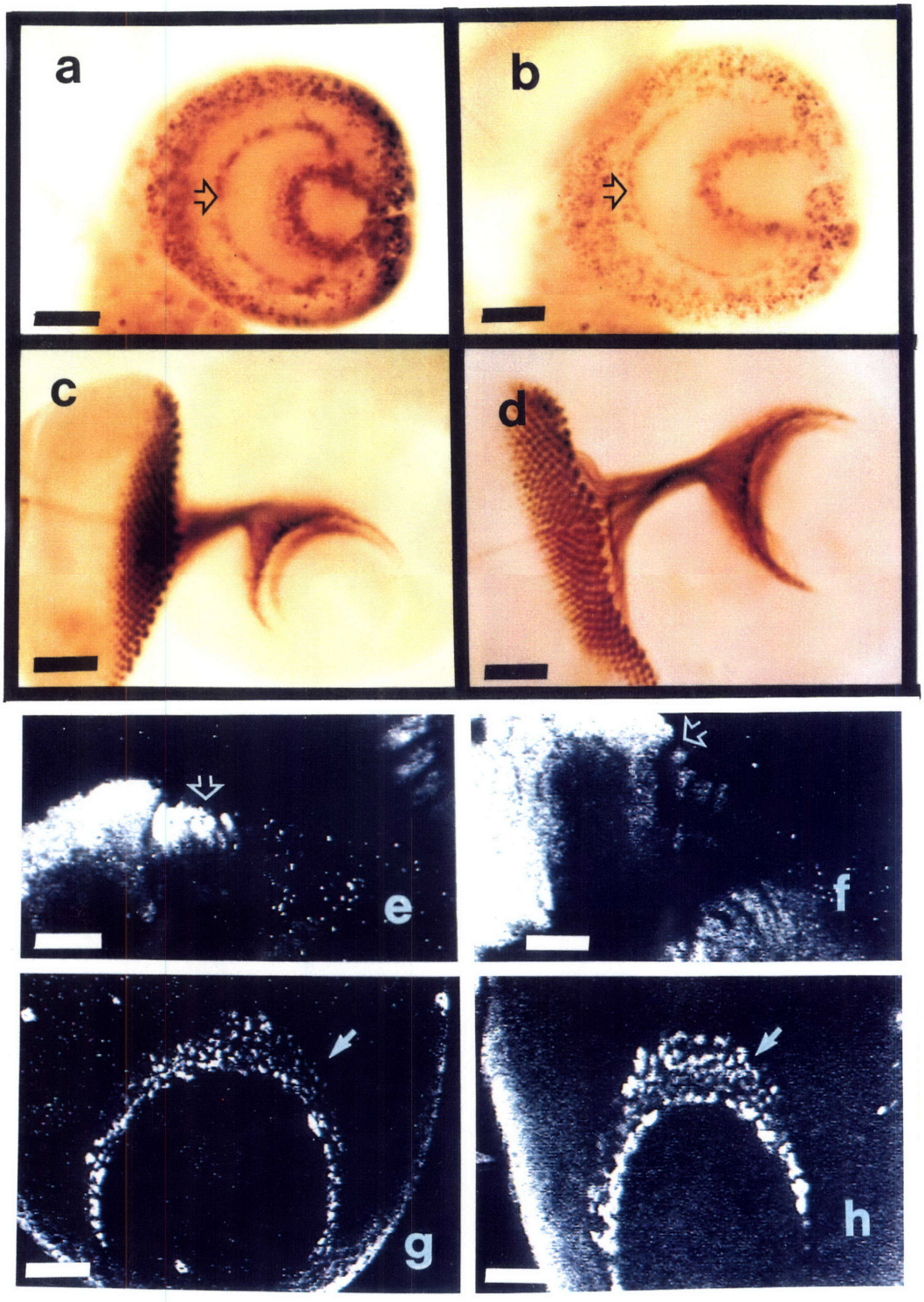

a
b
c
d
e
f
g
h

Figure 11. Analysis of the retina and lamina structure in *lama* adults.

Anterior is to the left. Semithin plastic sections of (A) ry^{506} and (B) $lama^{410}$ are stained with methylene/toluidine blue. The lamina cortex (arrowhead) and neuropil (arrow) is indicated in each section. Scale bars: 14 μm.

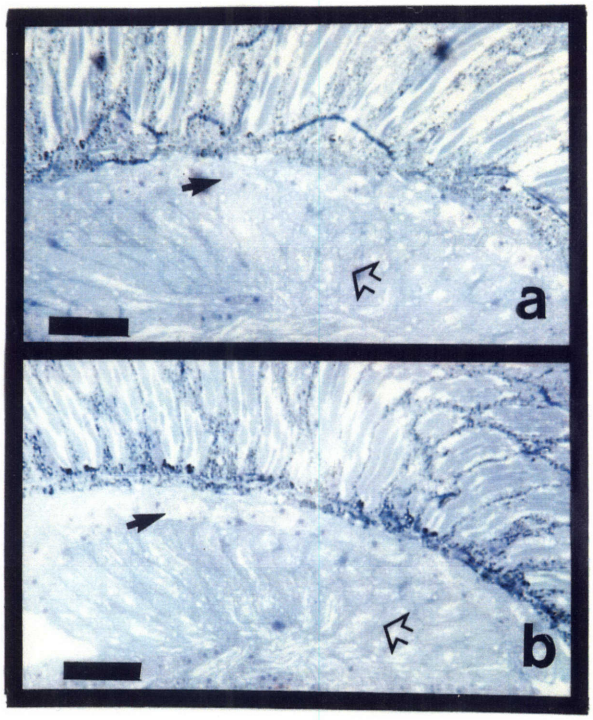

a
b

Figure 12. Sequence comparison of the *D. melanogaster* and *D. virilis lama* proteins.

A *D. virilis lama* homologue was isolated from a genomic library. The homology to *lama* is found within a potential ORF in the *D. virilis* clone. The putative proteins are 74% identical over their entire lengths. Conserved residues are indicated with a vertical slash. Sequence similarity between the clones is disrupted in the 5' region by a consensus splice acceptor site in the *D. virilis* clone. Motifs 1 and 2 are underlined.

D. melanogaster 1 *M*ERPEYDGTYCATALWTKQVGFQIENWKQQNDLVNIPTGVGRI 43

44 CYKDSVYENGWAQIEVETQRTYPDWVQAYAAGMLEGSLTWRNIYNQWSN..... 92

D. virilis 1 WAQIEVETQRSYPDWVQAYAAG I LEGSLTWKNIYNQWAKKYEFSSLATHS 50

93 .....TISSSCERDESTQKFCGWLRDLLTTNYHRLKRQ...TEKAEN 131

51 YSLNFLPALSTISSSCDRDESSQKFCECVRNLLTRNYEQLKEQAAAMAEH 100

132 DHYWHQLHLFITQLEGL ETGYKRGASRARSDLEEEIPFSDFLLMNAAADI 181

101 DHYWHQLHLILNQLEGMETGYI RGATRARSDLEEEIPLSDFLLMNAAADI 150

182 QDLKIYYENYELQNSTEHTEEPGTDQPKNFFLPSATMLTKIVQEEESPQ 230

151 QDLKIYYENYVLPNGSAECGRQAG..SKNFFLPSASMLTRLLRDEQAPQQ 198

231 VLQLLFGHSTAGSYSSMLRIQKRYKFHYHFSSKLRSNTVPGVDITFTGYP 280

199 SLQLLFGHSTAGSYSSMLRIQKRYKFHYHFSPDTRSNTVPGADITFTGYP 248

281 GILGSTDDFYTI KGRHLHAIVGGVGIKNENLQLWKTVDPKKMVPLVARVM 330

249 GIIGSTDDFYVVKGRQVQSIVGGVCIKNENLALWEKVDVKNMVPLVARVM 298

331 AANRISQNRQTWASAMSRHPFTGAKQWITVDLNKMKVQDNLYNVLEGDDK 380

299 AANRIAQNRRTWARAMSRHPFTGAKQWISVDLNKLGAQDNLYNTLDADEK 348

381 HDDAPVVLNEKDRTAIQQRHDQLRDMVWIAEQLPGMMTKKDVTQGFLVPG 430

349 HDDAPVPLNEKDNAAISERHDQLKNMVWIVEQLPGL VHSKDVTEN FLLAG 398

431 NTSWLANGVPYFKNVLELSGV.....NYSEDQQLT 460

399 NSTWLANGVPYFDVILNASRISRDNYSEDQDLTPAEEAVPYNYSEDQDLT 448

461 VADEEELTSLASVDKYLRTHGFRGDLLGSQESIAYGNIDLK. LFSYNARL 509

449 PAEEAELTNLEAVDKYLRNGGFRGDLLG.DESIAYGNIDLKCYFSYNARL 497

510 GI..SDFHAFAGPVFLRFQHTQPRT...LEDEGQ....DGGVPPAASMGDERLSVSI 557

498 GMSDYHAFAGPI FLRLQHAQARSSLEEPQLDQADQAAPAA....IGDERLSVTI 547

558 EDADSLAEMELITERRSVRNDMRAIAMRKIGSGPFKWSEMSPVEEGGGHE 607

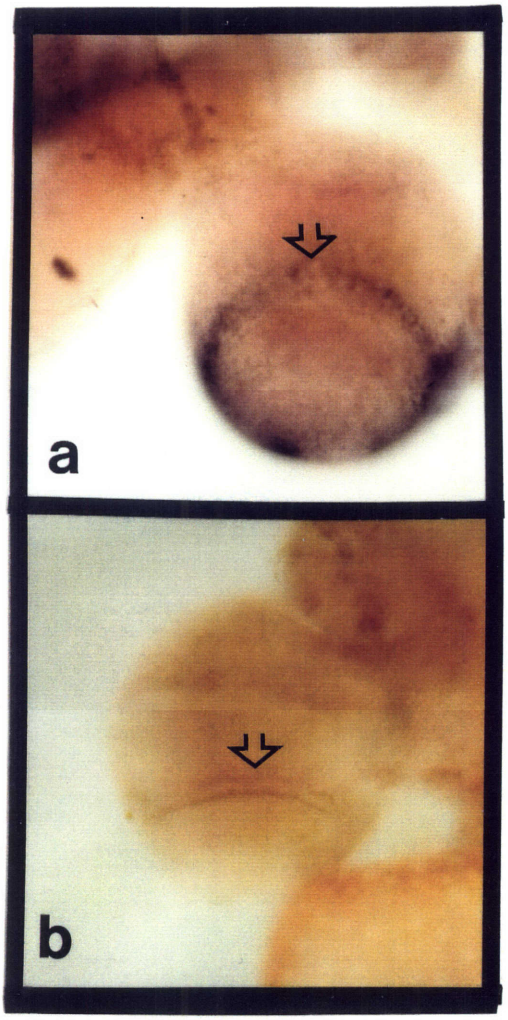
548 DDAHTLAELELITERRPVRNDMRAIAMRKIGSGPFKWSAMS..VLDDGN HA 596

608 GHPDEWNFDKVSPKWAW* 624

597 GHPDEWNFDKVSPRWVVSVPIVLCYVPHRISPATTATEKQ* 637

Figure 13. Expression pattern of *D. virilis lama*

lama expression is detected via whole mount *in situ* hybridization. (A) Expression of *lama* in *D. melanogaster*. (B) Expression of *lama* in *D. virilis*.


a
b

V. Future studies

Examining the role of glia in the development of the first optic ganglion

By analogy to other systems, glia within the lamina (Fig. 1) have the potential to affect the ganglion's development in a variety of ways. The lamina glia (L-glia) develop earlier than the lamina neurons (L-neurons), and are required in adult animals for their survival (Xiong and Montell, 1995 and see Fig. 2). At earlier stages, however, the L-glia are in the correct positions within the tissue to interact with both ingrowing retinal axons and with developing L-neurons.

(1) Retinal axons terminate in one of two optic ganglia. The outer photoreceptors (R1-6) project to the lamina, while the inner photoreceptors (R7,8) project to the medulla. The L-glia which flank the R1-6 termini may provide them with a specific termination signal. Differential pathfinding in response to local cues has already been demonstrated in the vertebrate optic chiasm (Stretavan et. al., 1993) and in the optic tectum (Johnston et. al., 1991; Drescher et. al., 1995).

(2) L-glia are closely associated with developing L-neurons, and could trigger neuronal differentiation, in a manner reminiscent of glia at the *Drosophila* midline (Klaes et. al., 1994).

(3) The lamina furrow generates L-neurons as well as cells which lie between the neuronal and glial cell bodies. It is presumed that these cells degenerate, because they are not found at the posterior border of the developing tissue, or in the adult animal (Winberg and Steller, unpublished observations; Winberg et. al., 1992). The L-glia may provide a limiting trophic factor, which allows only a subset of the furrow's daughters to survive. Alternatively, the L-glia may actively induce apoptosis in the non-neuronal cells. These mechanisms are used in the vertebrate retina (Armson et. al., 1987) and at the *Drosophila* midline (Sonnenfeld and Jacobs, 1995).

Ablation of the L-glia would test the hypothesis that the cells are required for some aspect of the lamina's development. Previous ablation experiments have relied on access to well defined promoters hooked up to a toxic compound (e.g.. Kunes and Steller, 1991; Grether et. al., 1995). The developmet of the GAL-4 system alleviated the need for characterized promoters (Brand and Perrimon, 1993). Unfortunately, it is not trivial to identify loci specifically expressed in the L-glia. P element enhancer trap screens rarely identify loci with expression restricted to one cell type within the third instar brain (Steller lab, unpublished observations; Datta et. al., 1992). Genes expressed in the developing L-glia often

show embryonic expression as well, making it unlikely that expression of a toxin could be tightly controlled. Recently, however, Lin and colleagues designed a stage specific, "blue death" ablation system (Lin et. al., 1995).

In the "blue death" system, experimental animals carry three transgenes (Fig. 3). The first transgene contains a "masked" diphtheria toxin under UAS control. The "mask" consists of a *lacZ* reporter flanked by FRT target sites. The second transgene contains GAL-4 under a minimal promoter, and is inserted near an enhancer which drives GAL-4 in the pattern of interest. In the presence of a GAL-4 insertion and the masked toxin, *lacZ* is expressed in a restricted pattern. Animals carry a third transgene which consists of the FLP recombinase under control of the heat shock promoter (Golic and Lindquist, 1989; Struhl and Basler, 1993). Following application of a mild heat pulse, a small amount of FLP recombinase is expressed. In some fraction of cells, the "mask" is excised from the toxin coding sequence and cells begin to express the toxin in place of *lacZ*. The GAL-4 insertion provides spatial specificity of toxin expression, while the selective application of a heat pulse provides temporal specificity. If the appropriate GAL-4 lines are available, ablation of the L-glia would be trivial.

Another way to determine the developmental function of the L-glia would be to identify a mutation which blocks their migration and/or their differentiation. Many mutations which block development of the embryonic midline glia have been identified (see Klämbt et. al., 1991). One well characterized locus is *pointed* (*pnt*), which encodes two related ETS transcription factors (Klambt, 1993). Glia at the midline do not develop properly in *pnt* mutants, and, as a consequence, fail to migrate. *pnt* also functions in photoreceptor development (O'Neill et. al., 1994). In the embryo, ectopic expression of one of the *pnt* transcripts is sufficient to induce ectopic glial cells (Klaes et. al., 1994). The ectopic glia subsequently induce the differentiation of ectopic neurons.

pnt's expression pattern suggests that it may also be required for the development of the L-glia (Perez and Steller, unpublished observations). *pnt* is expressed in both the lamina's neuronal and glial precursors, as well as in the most superficial layer of mature L-glia (arrowhead) (Fig. 4).

A preliminary analysis of hypomorphic *pnt* alleles indicate that the gene's function is required for normal retinal innervation of the brain. Two different alleles were examined for their effects on retinal projections. One allele disorders

the termination of R1-6 (Fig. 5A). Another allele appears to cause the axons to remain in a tangle on the brain's surface (Fig. 5B). Although this retinal projection defect is consistent with a defect in the L-glia, it will be necessary to separate *pnt's* function in the retina from its putative function in the lamina. Several *pnt* alleles are already available on FRT chromosomes, which should make a mosaic analysis straightforward (O'Neill et. al., 1994).

Further genetic analyses of *lama*

One way to find a *lama* phenotype in the developing visual system is to examine the effects of *lama* mutations in a sensitized background. Candidates for a double mutant analysis may be identified based on their expression pattern in the third instar brain. One candidate, *pnt*, has been described above.

A number of other genes are expressed in a pattern that overlaps *lama's* expression, and would be good candidates for a double mutant analysis. The *Drosophila* EGF (DER) receptor (Zak and Shilo, 1992) and two of the neurogenic genes, *Notch* (*N*) and *neuralized* (*neu*), are expressed in the lamina neuronal precursors (Johansen, et. al., 1989 ; Boulianne, et. al., 1991). Although roles for DER and *N* in the development of the visual system have been described (Baker and Rubin, 1989; Baker and Rubin, 1992; Markopoulou, et. al., 1989), it is not clear if, or how, these genes autonomously affect the development of the lamina. One of the proneural genes, *asense*, (Gonzalez, et. al., 1989), and three homologues of bone morphogenetic proteins (BMP), *tolloid*, *tolloid-related-1*, and *tolkin* (Nguyen, et. al., 1994; Finelli, et. al., 1995) appear to be expressed in both the neuronal and glial precursors. The expression of *tartan*, a cell surface protein, overlaps with *lama* in both the embryo and the third instar brain (Chang, et. al. ,1993). Interestingly, both *tartan* and *tolloid* appear to be in a pathway with *decapentaplegic* (*dpp*) (Chang, et. al., 1993; Childs and O'Connor, 1994). A requirement for *dpp* in the development of the retinal axon target field has been previously demonstrated (Kaphingst and Kunes, 1994). It is possible that *lama* functions with one or more of these similarly expressed genes.

Another way in which to identify a double mutant candidate would be to make use of the fact that the *lama* ORF detects related sequences on *D. melanogaster* genomic Southerns probed at low stringency. Preliminary low

stringency screens of a genomic library indicate that it should be relatively straightforward to isolate a *lama* homologue (Perez and Steller, unpublished observations)

References

Armson, P., Bennet, M. R. and Raju, T. (1987). Retinal ganglion cell survival and neurite regeneration requirements: the change from Müller cell dependence to superior colliculi dependence during development. *Dev. Brain Res.* **32:** 207-216.

Baker, N. E. and Rubin, G. M. (1992). *Ellipse* mutations in the *Drosophila* homologue of the EGF receptor affect pattern formation, cell divisions and cell death in eye imaginal discs. *Dev. Biol.* **150**: 381-396.

Baker, N. E. and Rubin, G. M. (1989). Effect on eye development of dominant mutations in *Drosophila* homologue of the EGF receptor. *Nature.* **340:** 150-153.

Bouillianne, G. L., de la Concha, A., Campos-Ortega, J. A., Jan, L. Y. and Jan, Y. N. (1991). The *Drosophila* neurogenic gene *neuralized* encodes a novel protein and is expressed in the precursors of larval and adult neurons. *EMBO J.* **10:** 2975-2966.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and dominant phenotypes. *Dev.* **118:** 401-415.

Campbell, G., Goring, H., Lin, T., Spana, E., Andersson, S., Doe, C. Q., and Tomlinson, A. (1994). RK2, a glial-specific homeodomain protein required for embryonic nerve cord condensation and viability in *Drosophila*. *Dev.* **120:** 2957-2966.

Chang, Z., Price, B. D., Bockheim, S., Boedigheimer, M. J., Smith, R. and Laughon, A. (1993). Molecular and genetic characterization of the *Drosophila tartan* gene. *Dev. Biol.* **160:** 315-332.

Childs, S. R. and O'Connor, M. B. (1994). Two domains of the tolloid protein contribute to its unusual genetic interaction with *decapentaplegic*. *Dev. Biol.* **162:** 209-220.

Datta, S., Stark, K. and Kankel, D. R. (1993). Enhancer detector analysis of the extent of genomic involvement in nervous system development in *Drosophila melanogaster*. *J. Neurobiology*. **24:** 824-841.

Drescher, U., Kremoser, C., Handwerker, C., Loschinger, J., Noda, M., and Bonhoeffer, F. (1995). *In vitro* guidance of retinal ganglion cell axons by RAGS, a 25 kDa tectal protein related to ligands for Eph receptor tyrosine kinases. *Cell.* **82:** 359-370.

Finelli, A. L., Xie, T., Bossie, C. A., Blackman, R. K., and Padgett, R. W. (1995). The tolkein gene is a tolloid/BMP-1 homologue that is essential for *Drosophila* development. *Genetics*. **141:** 271-281.

Golic, K.S. and S., Lindquist. (1989). The FLP recombinase of yeast catalyzes site specific recombination in the *Drosophila* genome. *Cell*. **59:** 499-509.

Gonzales, F., Romain, S., Cubas, P., Modolell, J. and Campuzano, S. (1989). Molecular analysis of the *asense* gene, a member of the achaete-scute complex of *Drosophila melanogaster*, and its novel role in optic lobe development. *EMBO J.* **8:** 3553-3562.

Grether, M. E., Abrams, J. A., Agapite, J. A., White, K., and Steller, H. (1995). The *head involution defective* gene of *Drosophila melanogaster* functions in programmed cell death. *Genes and Dev.* **9:**1694-1708.

Johansen, K. M., Fehon, R. G. and Artavanis-Tsakonas, S. (1989). The *Notch* gene product is a glycoprotein expressed on the cell surface of both epidermal and neuronal precursor cells during *Drosophila* development. *J. Cell Biol.* **109:** 2427-2440.

Johnston, A. R. and Gooday, D. J. (1991). *Xenopus* temporal retinal neurites collapse on contact with glial cells from caudal tectum in vitro. *Dev.* **113:** 409-417.

Kaphingst, K. and Kunes, S. K. (1994). Pattern formation in the visual centers of the *Drosophila* brain: wingless acts via decapentaplegic to specify the dorsoventral axis. *Cell.* **78:** 437-448.

Klaes, A., Menne, T., Stollewerk, A., Schols, H., and Klämbt, C. (1994). The ETS transcription factors encoded by the *Drosophila* gene pointed direct glial cell differentiation in the embryonic CNS. *Cell.* **78:** 149-160.

Klämbt, C., Jacobs, R. J., and Goodman, C. S. (1991). The midline of the *Drosophila* CNS: a model for the genetic analysis of cell fate, cell migration, and growth cone guidance. *Cell.* **64:** 801-815.

Klämbt, C. (1993). The *Drosophila* gene pointed encodes two ETS-like proteins, which are involved in the development of the midline glial cells. *Dev.* **117:** 163-176.

Kunes, S. and Steller, H. (1991). Ablation of *Drosophila* photoreceptor cells by conditional expression of a toxin gene. *Genes and Dev.* **5:** 970-983.

Lin, D. M., Auld, V. J., and Goodman, C. S. (1995). Targeted neuronal cell ablation in the *Drosophila* embryo: pathfinding by follower growth cones in the absence of pioneers. *Neuron.* **14:** 707-715.

Markopoulo, K., Welshons, W. J. and Artivanis-Tsakonas, S. (1989). Phenotypic and molecular analysis of the *facets*, a group of intronic mutations at the *Notch* locus of *Drosophila melanogaster* which affect postembryonic development. *Genetics.* **122:** 417-428.

Nguyen, T., Jamal, J., Shimell, M. J., Arora, K. and O'Connor, M. B. (1994). Characterization of tolloid-related-1: a BMP-1 like product that is required during larval and pupal stages of *Drosophila* development. *Dev. Biol.* **166:** 569-586.

O'Neill, E. M., Rebay, I., Tjian, R. and Rubin, G. M. (1994). The activities of two ets-related transcription factors required for Drosophila eye development are modulated by the Ras/MAPK pathway. Cell. **78:** 137-147.

Stretavan, D. W. and Reichardt, L. F. (1993). Time-lapse video analysis of retinal ganglion cell axon pathfinding at the mammalian optic chiasm: growth cone guidance using intrinsic chiasm cues. *Neuron.* **10:** 761-777.

Struhl, G. and Basler, K. (1993). Organizing activity of wingless protein in *Drosophila. Cell.* **115:** 903-911.

Sonnenfeld, M. J. and Jacobs, J. R. (1995). Apoptosis of the midline glia during *Drosophila* embryogenesis: a correlation with axon contact. *Dev.* **121:** 569-578.

Winberg, M. L., Perez, S. E. and Steller, H. (1992). Generation and early differentiation of glial cells in the first optic ganglion of *Drosophila melanogaster. Dev.* **115:** 903-911.

Xiong, W-C. and Montell, C. (1995). Defective glia induce neuronal apoptosis in the *repo* visual system of *Drosophila. Neuron.* **14:** 581-590.

Zak, N.B. and Shilo, B-Z. (1992). Localization of DER and the pattern of cell divisions in wild type and ellipse eye imaginal discs. *Dev. Biol.* **149,** 448-456.

Figure 1. Schematic diagram of the developing lamina and putative roles for the lamina glia.

A horizontal view of the third instar lamina is shown with anterior to the left. Photoreceptor axons (R-axons) terminate in either the lamina (R1-6) or the medulla (R7,8). The lamina neurons (L-neurons) are indicated by filled circles. A superficial layer of lamina glia (L-glia, filled rectangles) is found beneath the L-neurons, while the deeper L-glia flank the R1-6 termini (arrow). The glia which surround the lamina (rectangles) are also shown. The furrow (ovals) is indicated with an arrowhead, and the non-neuronal furrow daughters are shown as circles.

The deep layers of L-glia are in the correct position to provide a stop signal to the R1-6 axons, preventing them from entering the medulla. The superficial layer of L-glia are adjacent to most of the L-neurons, and could potentially induce their differentiation. The L-glia could also be involved in the elimination of the non-neuronal cells that emerge from the furrow.

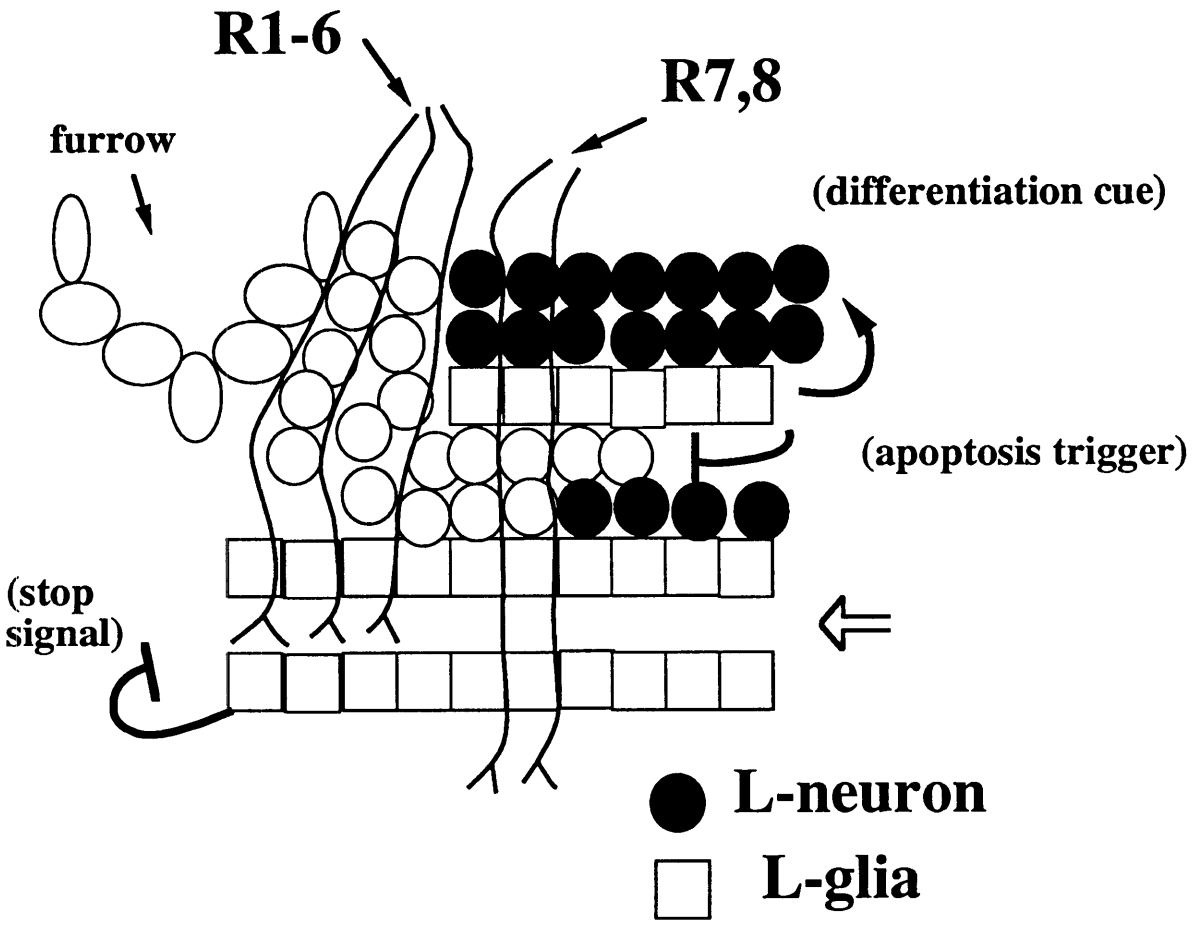

R1-6
R7,8
furrow
(differentiation cue)
(apoptosis trigger)
(stop
signal)
L-neuron
L-glia

Figure 2. Time course of neural-glial interactions within the *Drosophila* first optic ganglion.

Timeline depicts *Drosophila* development from the end of the first instar. Age is given as hours after egg laying (AEL). Interactions that have been demonstrated experimentally are indicated in bold, while putative interactions are indicated with open type. Data is summarized from Selleck and Steller, 1991; Winberg et. al., 1992; Meinertzhagen and Hanson, 1993; Perez and Steller, submitted; and Xiong and Campbell, 1995.

46 hours: Glial and neuronal progenitors have become distinct from each other.

74-90 hours: Glial progenitors complete their divisions, and immature glia begin to migrate into the lamina anlage.

94 hours: Retinal innervation begins, triggering neurogenesis and the continued migration and differentiation of the glial cells.

94-132 hours: Glia provide differential termination signal to retinal axons, and regulate the development of the neurons

132 hours: retinal innervation of the brain is complete.

160 hours: electrical activity begins in the lamina.

238 hours: eclosion.

post-eclosion: Integrity of the glial cells required for the survival of both lamina neurons and photoreceptors.

Figure 3. Schematic of the "blue death" cell ablation system

Lin and colleagues (Lin et. al., 1995) developed an ablation system that allows the targeting of very specific cell types. The system involves three transgenes. The first transgene (A) contains a masked diphtheria toxin gene under the transcriptional control of a UAS. The toxin is masked by insertion of a *lacZ* gene, flanked by FRT target sites. The second transgene (B) consists of GAL-4 placed under a minimal promoter. This transgene is inserted next to an enhancer which drives GAL-4 expression in a relevant pattern. The third transgene (C) is comprised of the FLP recombinase under control of the heat shock promoter.

In order to ablate the L-glia, the relevant insertion of transgene (B) should express GAL-4 in the lamina glia (L-glia) and their precursors. Animals carrying all three transgenes should be aged to late second or early third instar, and subjected to a mild heat pulse to induce a small amount of the FLP recombinase. Animals could then be aged and examined for loss of some of the L-glia. These animals could, simaltaneously, be analyzed for errors in the retinal axon projections, development of the L-neurons or persistance of the non-neuronal cells.


(A)
UAS
diptheria
lacZ
toxin


(B)
enhancer
GAL-4
(glial cell enhancer)


(C)
heat shock promoter
FLP recombinase


FRT target site

Figure 4. Expression of *pointed* in third instar brains.

Third instar brains were dissected from animals carrying the pnt^{7825} P element insertion (O'Neill et. al., 1994). The brains were stained for X-gal activity as previously described (chapter 4). *pnt* expression in the lamina neuronal precursors (arrowhead) is down regulated earlier than *llama* expression (compare to chapter 4, Fig. 1C). *pnt* is seen in the glial precursors (arrows) and in some of the glia within the lamina (open circles).

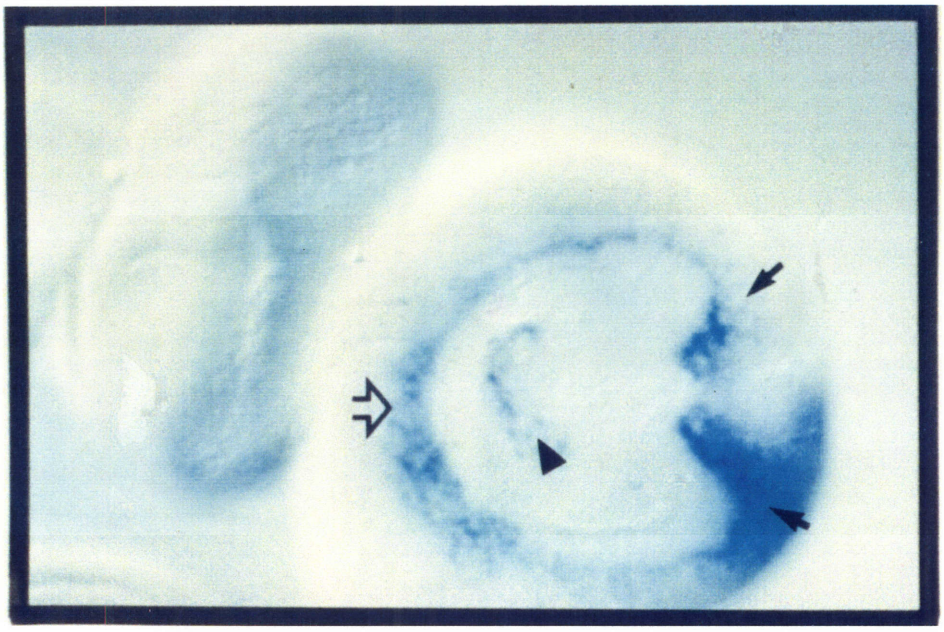


Figure 5. Hypomorphic alleles of *pnt* disrupt retinal axon projections.

Larval brains were dissected from (A) $pnt^{3680D21}$ and (B) $pnt^{7825D82}$ mutants (O'Neill et. al., 1994). Retinal axon projections were examined using a photoreceptor specific antibody, MAb 24B10, as previously described (chapter 4). Anterior is to the left in each panel. (A) Photoreceptor axons appear to be tangled on the brain's surface (compare to chapter 4, Fig. 9C). (B) Photoreceptor termini appear gapped (compare to chapter 3, Fig. 1B).


a
b

Appendix I. Initial characterization of a novel member of the *Drosophila* kinesin family, KLP64D.

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This work was done in collaboration with members of the Goldstein lab. KLP64D was first identified by Stewart et. al, 1991, and was independently isolated as a result of a P element enhancer trap screen for novel loci involved in the development of the *Drosophila* visual system (see chapter 4). The sequence of the cDNA was provided by the Goldstein lab. I determined the genomic structure of the locus, and examined the gene's expression pattern in embryos and in third instar larvae. Additionally, I generated P element excision mutations which delete into KLP64D, and performed a preliminary phenotypic analysis of KLP64D mutants.

SUMMARY

The study of motor proteins in genetic systems has provided insight into the functions of several of these molecules. We describe a novel member of the kinesin gene family, KLP64D. KLP64D is expressed in both the central and peripheral nervous systems of the embryo. Mutations in the locus appear to be lethal, although they do not obviously disrupt nervous system morphology.

INTRODUCTION

Three superfamilies of ATP dependent motor proteins have been identified: myosins, kinesins, and dyneins (reviewed in Endow and Titus, 1992; Brady, 1991). Work from a variety of organisms has indicated that myosin moves along actin filaments, while kinesin and dynein carry out fast axonal transport along microtubules.

Mutations in *Drosophila* motor proteins have implicated them in functions as diverse as cell division (McDonald and Goldstein, 1990; Zhang et. al., 1990; Heck et. al., 1993), endocytosis (van der Bliek and Meyerowitz, 1991), and intracellular transport (Montell and Rubin, 1989). Mutations in *Drosophila* kinesin (Khc) are lethal (Saxton et. al., 1991) and disrupt synaptic function (Gho et. al., 1992).

Kinesin like proteins (KLPs) have been isolated from many species and share homology with the motor domain of the kinesin heavy chain (Stewart et. al., 1991; Endow and Hatsumi, 1991; Aizawa et. al., 1992; Cole et. al., 1993 and reviewed in Goldstein, 1993). The structures of the KLPs vary outside of the motor domains, presumably reflecting functional differences.

We report the initial characterization of a novel *Drosophila* kinesin like protein, KLP64D. Sequence of a portion of the motor domain has been described previously (Stewart et. al., 1991). KLP64D shares considerable homology with another *Drosophila* KLP, KLP68D (Pesavento et. al., 1994). KLP64D homologues have been identified in two other organisms. A murine homologue, Kif 3, is expressed in the adult cerebellum and hippocampus (Akizawa et. al., 1993). A homologue in *S. purpuratus* encodes an 85 kD protein, which is part of a heterotrimeric complex in the sea urchin embryos (Cole et. al., 1993).

KLP64D is expressed in both the embryonic and larval nervous systems.

While deletions extending into the KLP64D transcript appear to be larval lethal, they do not appear to disrupt the architecture of the central or peripheral nervous systems.

MATERIALS AND METHODS

Stocks.

All stocks were maintained on a standard cornmeal medium at 18°C or 25°C. *Canton S* served as the wild type strain. P element insertions were generated as previously described (Winberg et. al., 1992). The L733 insertion, a gift of Dr. Ron Davis, is an insertion of the PlArb element (Wilson et. al., 1989). All insertions were mapped by *in situ* hybridization to chromosomes (Pardue, 1986), using pBluescript (Stratagene) as a probe to detect the lacZ reporter sequence.

Isolation of genomic clones and cDNAS.

A plasmid rescue (Steller and Pirotta, 1986) was obtained from the L733 line, and was used to screen a genomic library (gift of Dr. Ron Davis) by standard methods (Sambrook et. al., 1989). The cytological location of isolated genomic DNA was verified by using a lambda clone as a probe against a Canton S polytene chromosome squash (see above). A third instar larval CNS cDNA library (Wilson et. al., 1993) and a 9-11 hour embryonic cDNA library (Zinn et. al., 1988) were screened by standard methods using fragments of the genomic clones as probes. Two distinct classes of cDNAs were identified. The longest cDNAs were chosen for further analysis. Each cDNA detects a single transcript by Northern analysis.

Partial sequence of one cDNA indicated that it contains the previously reported KLP64D motor domain (Stewart et. al., 1991). KLP64D, originally named KLP4, was renamed to reflect its cytological location. Additional KLP64D cDNAs were obtained in a screen of an embryonic cDNA library.

The other cDNA encodes a novel gene, *lama* (see chapter 4).

Sequence analysis

Four KLP64D cDNAs and one corresponding genomic clone were sequenced using Circumvent (New England Biolabs) or Sequenase (United States Biochemical) sequencing kits. Sequence analysis was performed with software from the Genetics Computer Group (Madison, WI).

In situ hybridization to whole mount tissue

Two types of digoxigenein probes were made from the KLP64D cDNA. Hybridization of an antisense riboprobe to whole mount embryos was carried out as previously described (Tautz and Pfeifle, 1989), with modifications suggested by R. Bodmer (personal communication). Hybridization of a random primed DNA probe to the third instar larval CNS was carried out essentially as previously described (Zak and Shilo, 1992), except that RNA:DNA hybrids were detected with an anti-digoxigenin antibody coupled to an alkaline-phosphatase moiety.

Excision mutagenesis

Small deletions were generated in the region by imprecise excision of the P elements. Animals of the PZA8, PZA5, or PlArbL733 lines were crossed to animals carrying Δ2.3Sb, a chromosome bearing a stable, somatic source of the P transposase (Laski et. al., 1986; Robertson et. al., 1988). Animals that were genotypically P[ry+]/D2.3Sb were crossed to mhw,r,e/TM3Sb,ry^{RK}. Progeny were scored for loss of the ry^+ marker, and ry^- animals were crossed to CxD/TM3Sb to establish stable lines. The TM3Sb chromosome is marked with an *armadillo-lacZ* transgene (gift of L. Y. and Y. N. Jan), enabling heterozygous animals to be detected via an X-gal stain. Lethal mutations were mapped using a deficiency for the region, Df(3L)ems-13, which uncovers 64B1-2/64E (gift of the Fristrom lab).

Embryo collections of several of the lethal mutations were examined by *in situ* hybridization using cDNAs from both of the transcripts in the region as probes. Deletions were characterized using a combination of Southern analysis and PCR.

Immunohistochemistry

Immunohistochemistry was carried out as previously described (Ashburner, 1989; Winberg et. al., 1992). The proliferation in third instar larval brains was analyzed by pulse labeling with BrdU as previously described (Winberg et. al., 1992). BrdU was detected with an anti-BrdU antibody (Becton Dickinson).
Retinal innervation of third instar larval brains were analyzed using a photoreceptor specific antibody, MAb24B10 (Zipursky et. al., 1984; gift of S. Benzer). The development of neurons in the first optic ganglion was analyzed using an antibody specific for neuronal nuclei, MAb44C11 (Robinow et. al., 1988, Bier et. al., 1988; gift of Y. N. and L. Y. Jan). The larval CNS was analyzed using a

general axonal marker anti-HRP (Jan and Jan, 1982; Cappell), anti-serotonin (YMC-1019 from Accurate Chemical and Science Corporation), and antibodies to neuroglian and neurotactin (anti-IB7 and anti-BP106 respectively, gifts of C. Goodman). Embryos were analyzed using MAb22C10 (gift of S. Benzer; Zipursky et. al., 1984) and anti-couch potato (gift of H. Bellen; Bellen et. al., 1992).

Antibodies were used at the following concentrations: anti-couch potato (1/1000); anti-Brdu (1/50); MAb24B10 (1/20); MAb22C10 and MAb 44C11 (1/5); anti-HRP (1/200); anti-serotonin(1/100); anti-neuroglian and anti-neurotactin (1/2). All secondary antibodies were used at 1/100: Goat anti-mouse (HRP), Goat anti-mouse (FITC) and Goat anti-rat (HRP).

RESULTS

Description of the KLP64D locus.

Three P element insertions in 64C11 were obtained (Fig 1A). Two transcription units, separated by 2.5 kB, were identified in the region. Corresponding cDNAs detect single transcript by Northern analysis (Stewart et. al., 1991; and data not shown). The KLP64D transcript is located 5' to the P insertions, and its 3' end is 780 bp from the H3 site (starred in Fig. 1A). The transcript located 3' to the insertions, *lama*, encodes a novel gene (see chapter 4).

Sequence analysis of KLP64D

The 2.4 kB KLP64D cDNA encodes a protein of 676 amino acids (Fig. 2). The amino terminus (residues 1-350; underlined in Fig. 2) is 42% identical to the kinesin motor domain (Yang et. al., 1989; Stewart et. al., 1991). A prediction of secondary structure indicates that residues 351 through 676 are likely to be alpha helical. KLP64D shares homology with several other KLPs throughout its motor domain and its coiled coil region. At the amino acid level, KLP64D is 53% similar to *Drosophila* KLP68D (Pesavento et. al., 1994) and 59% similar to the 85 kD subunit of an *S. purpuratus* complex (Cole et. al., 1993). KLP64D is 55% identical to its mouse homologue, Kif 3 (Aizawa et. al., 1992). Within its colied-coil region, KLP64D is 42% similar to KLP68D (italicized residues, Fig. 2). The KLP64D cDNA sequence is contiguous with the genomic clone, and a polyadenylation consensus site is found 20 bp upstream of the poly A tail.

Expression pattern of KLP64D

KLP64D was previously reported to be expressed throughout development, and in adult heads and ovaries (Stewart et. al., 1991). *In situ* hybridization indicates that KLP64D is observed around the periphery of the embryo during the cellular blastoderm stage (Fig. 3A), indicating a maternal contribution of the message. In older embryos, KLP64D expression is restricted to the nervous system. Expression in late embryos is seen in both the CNS and the PNS (Fig. 3B-3E). KLP64D is expressed in most cells of the ventral nerve cord (Fig. 3C) and in several of the peripheral sensory organs (Fig. 3D,E). The embryonic expression pattern of KLP64D is quite similar to that of the related protein, KLP68D (Pesavento et. al., 1994). Expression continues during larval life. In late third instar larvae, KLP64D is expressed in the ventral nerve cord (Fig. 3F), and in a restricted pattern in the brain.

KLP64D expression does not mirror the expression of the P element reporters. The βgalactosidase reporter is expressed in the same cell types as the neighboring transcript, *lama* (see chapter 4).

Generation of KLP64D mutations

In order to generate a KLP64D mutation, the P element lines were used as the basis of an excision mutagenesis (n=400). Seven lethal deletions in one complementation group was identified. All of these alleles extend from the P insertion site towards KLP64D. *lama* expression is not disrupted by these deletions.

The lethal deletions were analyzed by Southern analysis. Two alleles extend into the KLP64D ORF (A5.6b and A8.n123), and a third extends into the transcript (A5.92). Analysis via *in situ* hybridization indicates that these alleles disrupt KLP64D expression in the embryo. Four additional deletions have breakpoints between the KLP64D and *lama* transcripts. It is possible that these deletions remove 3' regulatory sequences that are essential for wild type KLP64D expression. Two of the smaller deletions were examined (L733.34, A8.72) via *in situ* hybridization. These alleles do not appear to disrupt KLP64D expression in the embryo. We would not, however, have detected subtle effects these deletions may have on KLP64D's embryonic expression, nor have we examined KLP64D expression in larvae from these mutant lines.

It is possible that the lethality associated with the deletions is not due to a disruption of KLP64 expression. We think that this is unlikely for the following reason. All of the lethal deletions remove the H3 site between the *lama* and

KLP64D transcripts (Fig. 1A). Smaller deletions, which lie between this H3 site and the *lama* transcript are viable. Therefore, a component of an additional, essential transcription unit must be confined to the 780 base pair region between the H3 site and KLP64D. This region does not detect a signal by Northern analysis (data not shown).

Analysis of KLP64D mutations

The KLP64D mutations are primarily larval lethal, with the occasional mutant surviving to pupal stages. KLP64D mutants were examined for nervous system defects. Our analysis was confined to the alleles which disrupt the KLP64D transcript.

We examined the structure of the embryonic PNS using two different markers. MAb22C10 detects peripheral axons (Zipursky et. al., 1984) and anti-couch potato detects peripheral cell bodies (Bellen et. al., 1992). In embryo collections of the A5.92 and A5.6b lines, the PNS appears indistinguishable from the wild type PNS. The placement of the neuronal cell bodies in the peripheral sense organs (n=20), and the outgrowth of their axons (n=40) appear normal.

Larvae homozygous for the A5.6b allele appear to have no gross disruptions of the ventral nerve cord or the brain hemispheres. In the late third instar brain, proliferation detected by an *in vitro* BrdU pulse (n=15), retinal innervation detected by MAb24B10 (n=3) and the neuronal cell bodies of the first optic ganglion (n=3) appear normal. The structure of the CNS was examined using antibodies to neurotactin (n=5), neuroglian (n=5) and serotonin (n=9). The structure of the CNS in mutants is indistinguishable from that seen in wild type animals.

DISCUSSION

We have identified a novel *Drosophila* kinesin like protein, KLP64D, which is expressed in the developing central and peripheral nervous systems. KLP64D shows extensive homology to another *Drosophila* KLP, KLP68D; a CNS specific murine KLP, Kif 3; and to the 85 kD subunit of a heterotrimeric complex in *S. Purpuratus* (Pesavento et. al., 1994; Aizawa et. al., 1992; Cole et. al., 1993). The homologous regions include the motor domains, and the alpha helical, coiled coil regions. In the *S. purpuratus* complex, the coiled-coil regions are thought to mediate intermolecular associations. This observation, in addition to the high

degree of homology between KLP64D and KLP68D, raises the possibility that the *Drosophila* molecules also function as part of a multimeric complex (reviewed in Goldstein, 1993).

Although deletions which extend into the KLP64D transcript are lethal, they do not obviously disrupt the structure of either the central or peripheral nervous systems. It is possible that KLP64D and KLP68D have redundant functions, and that a mutation in either is insufficient to disrupt the overall architecture of the nervous system. In this case, an examination of the nervous system in KLP64D, KLP68D double mutants may be informative.

KLP64D function may be better understood by analyzing mutations via genetic mosaics. Small patches of mutant tissue in otherwise wild type adults may be analyzed for structural abnormalities, and for, perhaps, an age dependent degeneration of mosaic patches, due to abnormalities in neuronal function. Given its expression pattern in the nervous system, it is reasonable to predict that KLP64D may be involved in axonal transport. KLP64D may have a role in neuronal function, as does the *C. elegans* unc-104 locus (Hall and Hedgecock, 1991), and not in the initial formation of axonal projections. As the localization of synaptic vesicles to neuronal termini is unaffected by mutations in *Drosophila* kinesin (Gho et. al., 1992), it is possible that one or more KLPs function in synaptic vesicle transport. An electron microscopy examination of axonal termini in KLP64D mutants would indicate whether or not this particular KLP fills that role.

REFERENCES

Aizawa, H., Sekine, Y., Takemura, R., Zhang, Z., Nangaku, M. and Hirokawa, N. (1992). Kinesin family in murine central nervous system. *J. Cell Biol.* **119:** 1287-1296.

Ashburner, M. (1989). In *Drosophila: A Laboratory Manual.* Cold Spring Harbor Laboratory, New York: Cold Spring Harbor Laboratory Press.

Bellen, H. J., Kooyer, S., D'Evelyn, D. and Pearlman, J. (1992). The *Drosophila couch potato* protein is expressed in nuclei of peripheral neuronal precursors and shows homology to RNA-binding proteins. *Genes and Dev.* **6:** 2125-2136.

Brady, S.T. (1991). Molecular motors in the nervous system. *Neuron.* **7:** 521-533

Bier, E., Ackerman, L., Barbel, S., Jan, L. Y. and Jan, Y. N. (1988). Identification and characterization of a neuron specific nuclear antigen in *Drosophila. Science.* **240:** 913-916.

Cole, D.G., Chinn, S.W., Wedaman, K.P., Hall, K., Vuong, T. & Scholey, J.M. (1993). Novel heterotrimeric kinesin-related protein purified from sea urchin eggs. *Nature.* **366:** 268-270.

Endow, S.A. & Hatsumi, M. (1991). A multimember kinesin gene family in *Drosophila. PNAS.* **88:** 4424-4427.

Endow, S.A. & Titus, M.A. (1992). Genetic approaches to molecular motors. *Annu. Rev. Cell Biol.* **8:** 29-66.

Gho, M., McDonald, K.., Ganetsky, B., & Saxton, W.M. (1992). Effects of kinesin mutations on neuronal function. *Science.* **258:** 313-316.

Goldstein, L.S.B. (1993). With apologies to Scheherazade: tails of 1001 kinesin motors. *Annu. Rev. Genetics.* **27:** 319-351.

Hall, D.H. & Hedgecock, E.M. (1991). Kinesin-related gene unc-104 is required for axonal transport of synaptic vesicles in *C. elegans. Cell.* **65:** 837-847.

Heck, M.M.S., Pereira, A., Pesavento, P., Yannoni, Y., Spradling, A.C. & Goldstein, L.S.B. (1993). The kinesin-like protein KLP61F is essential for mitosis in *Drosophila. J. Cell Biol.* **123:** 665-679.

Jan, L. Y. and Jan, Y. N. (1982). Antibodies to horseradish peroxidase as specific neuronal markers in *Drosophila* and grasshopper embryos. *PNAS.* **79:** 2700-2704.

Laski, F.A., Rio, D.C. & Rubin, G.M. (1986). Tissue specificity of *Drosophila* P element transposition is regulated at the level of mRNA splicing. *Cell.* **44:** 7-19.

McDonald, H.B. & Goldstein, L.S.B. (1990). Identification and characterization of a gene encoding a kinesin-like protein in *Drosophila. Cell.* **61:** 991-1000.

Mlodzik, M. & Hiromi, Y. (1992). The enhancer trap method in Drosophila: its application to neurobiology. In *Methods in Neuroscience, vol 9: Gene expression in neural tissues.* (ed. P. M. Conn). pp. 397-414. New York: Academic Press.

Montell, C. & Rubin, G.M. (1989). The *Drosophila ninaC* locus encodes two photoreceptor cell specific proteins with domains homologous to protein kinases and the myosin heavy chain head. *Cell.* **52:** 757-772.

Pardue, M.L. (1994). Looking at polytene chromosomes. In *Drosophila melanogaster: practical uses in cell and molecular biology.* (eds. L. S. B. Goldstein and E. A. Fryberg). pp. 219-239. New York: Academic Press.

Pesavento, P. A., Stewart, R. J. and Goldstein, L. S. (1994). Characterization of the KLP68D kinesin-like protein in *Drosophila*: possible roles in axonal transport. *J. Cell Biol.* **127:** 1041-1048.

Robertson, H.M., Preston, C.R., Phillis, R.W., Johnson-Schlitz, D.M., Denz, W.K. & Engels, W.R. (1988). A stable genomic source of P element transposase in *Drosophila melanogaster. Genetics.* **118:** 461-470.

Robinow, S., Campos, A. R., Yao, K. M. and White, K. (1988). The *elav* gene product of *Drosophila*, required in neurons, has three RNP consensus motifs. *Science.* **242:** 1570-1572.

Sambrook, J., Fritsch, E.F., & Maniatis, T. (1989). In *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, New York: Cold Spring Harbor Laboratory Press.

Saxton, W.M., Hicks, J., Goldstein, L.S.B. & Raff, E.C. (1991). Kinesin heavy chain is essential for viability and neuromuscular function in *Drosophila*, but mutants show no defects in mitosis. *Cell.* **64:** 1093-1102.

Steller, H. and Pirotta, V. (1986). P transposons controlled by the heat shock promoter. *Mol. Cell Biol.* **6:** 1640-1649.

Stewart, R.J., Pesavento, P.A., Woerpel, D.N. & Goldstein, L.S.B. (1991). Identification and partial characterization of six members of the kinesin superfamily in *Drosophila. PNAS.* **88:** 8470-8474.

Tautz, D. & Pfeifle, C. (1989). A non-radioactive *in situ* hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. *Chromosoma.* **98:** 81-85.

van der Bliek, A. M. & Meyerowitz, E. (1991). Dynamin-like protein encoded by the *Drosophila shibire* gene associated with vesicular traffic. *Nature.* **351:** 411-414.

Wilson, C., Pearson, R.K., Bellen, H.J., O'Kane, C.J., Grossniklaus, U. & Gehring, W.J. (1989). P element mediated enhancer detection: isolation and characterization of developmentally regulated genes. *Genes and Dev.* **3:**1301-1313.

Wilson, C., Goberdahn, D.C.I. & Steller, H. (1993). *Dror*, a potential neurotrophic receptor gene encodes a *Drosophila* homologue of the vertebrate Ror family of Trk-related receptor tyrosine kinases. *PNAS.* **90:** 7109-7113.

Winberg, M. L., Perez, S. E. and Steller, H. (1992). Generation and early differentiation of glial cells in the first optic ganglion of *Drosophila melanogaster. Dev.* **115:** 903-911.

Zak, N.B. & Shilo, B-Z. (1992). Localization of DER and the pattern of cell divisions in wild type and ellipse imaginal discs. *Development.* **114:** 113-123.

Zhang, P., Knowles, B.A., Goldstein, L.S.B. & Hawley, R.S. (1990). A kinesin-like protein required for distributive chromosome segregation in *Drosophila. Cell.* **62:** 1053-1062.

Zinn, K., McAllister, L., & Goodman, C.S. (1988). Sequence analysis and neuronal expression of *fasiclin I* in grasshopper and *Drosophila. Cell.* **53:** 577-587.

Zipursky, S., Venkatesh, T. and Benzer, S. (1984). From monoclonal antibody to gene for a neuron-specific glycoprotein in *Drosophila. PNAS.* **82:** 1855-1859.

Figure 1: Genomic region surrounding KLP64D

The genomic map of the 64C11 region is shown, and the P element insertions are represented by filled triangles. KLP64D and *lama* are represented as rectangles, coding sequences are indicated by filled rectangles, and the direction of transcription is indicated by arrows. Deletion mutations are drawn as filled bars underneath the map.

Five mutations in *lama* were generated by imprecise excision of PZA8. All *lama* mutations are viable and fertile. Deletions into *lama's* first exon are insufficient to disrupt transcript expression. $lama^{410}$ is a null allele which completely deletes the transcript.

Seven mutations in KLP64D were generated, and the P element insertion used to generate each allele is indicated by its name. KLP64D mutations are lethal and do not affect *lama's* expression. Three alleles, ΔA8.n123, ΔA5.6b and ΔA5.92, disrupt transcribed sequence. The breakpoints of the remaining alleles are confined to the region between KLP64D and *lama*. All KLP64D alleles remove the H3 site 3' to the transcript.

Deletions which retain part of the P elements are indicated with hatched rectangles. (R) Ecor1, (C) Cla1, (Sp) Spe1, (H) HindIII, (S) Sac1, (Hp) Hpa1, (E) Eag1, (P) Pst1, (B) Bamh1.

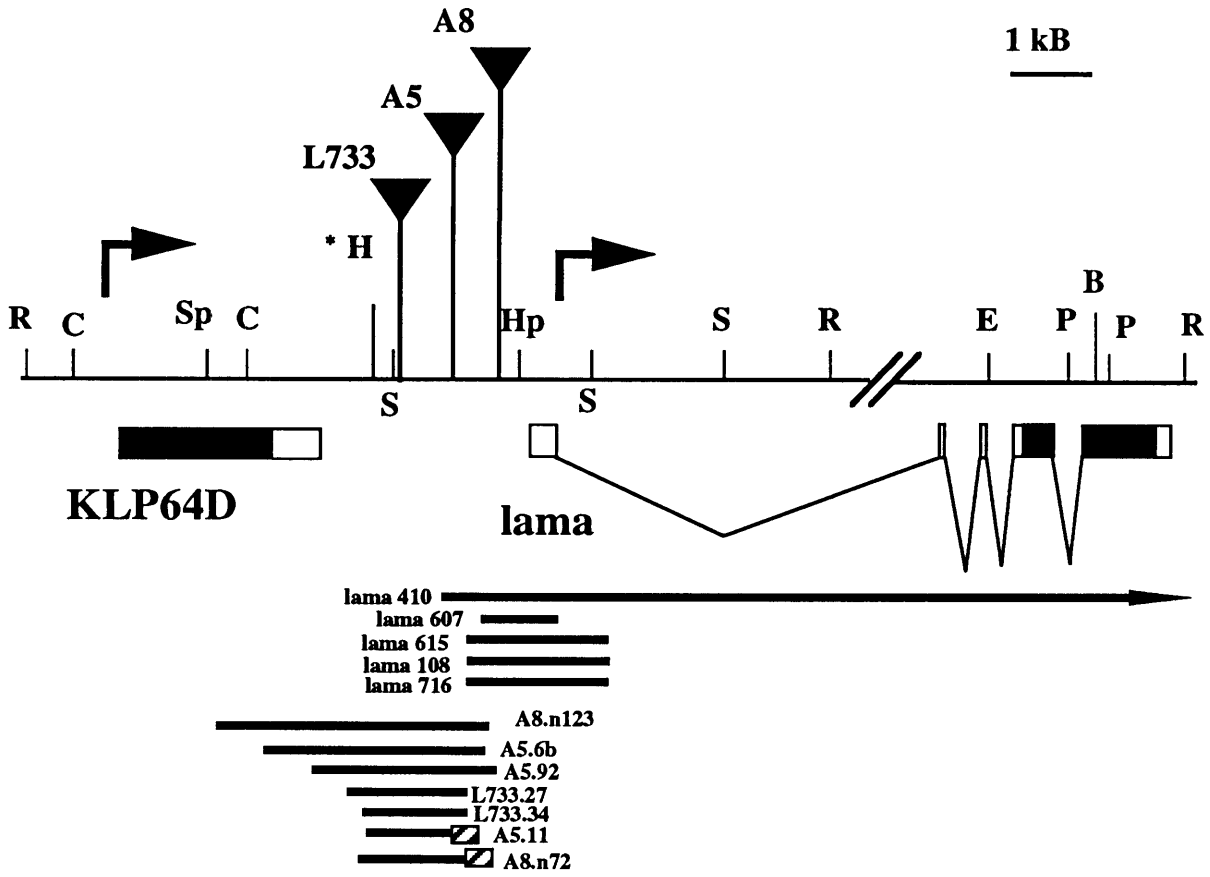

A8
A5
L733
* H
1 kB
R
C
Sp
C
Hp
S
R
E
P
B
P
R
S
S
KLP64D
lama
lama 410
lama 607
lama 615
lama 108
lama 716
A8.n123
A5.6b
A5.92
L733.27
L733.34
A5.11
A8.n72

Figure 2. Sequence of KLP64D

The sequence of a KLP64D cDNA is shown above the predicted amino acid sequence of the KLP64D ORF. Numbers to the left indicate base pair positions within the nucleic acid sequence, while numbers to the right indicate amino acid residues within the coding sequence. The putative motor domain is encoded by residues 1-350 (underlined residues). The putative coiled coil region is shown in italics. A poly-adenylation consensus site is shown in bold.

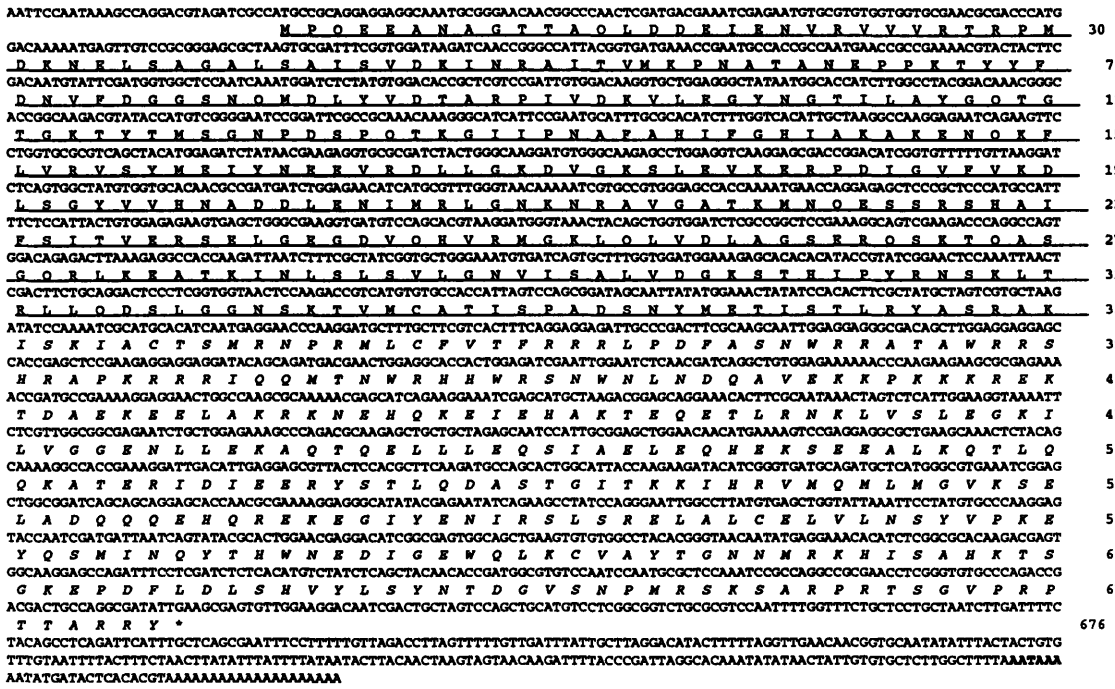
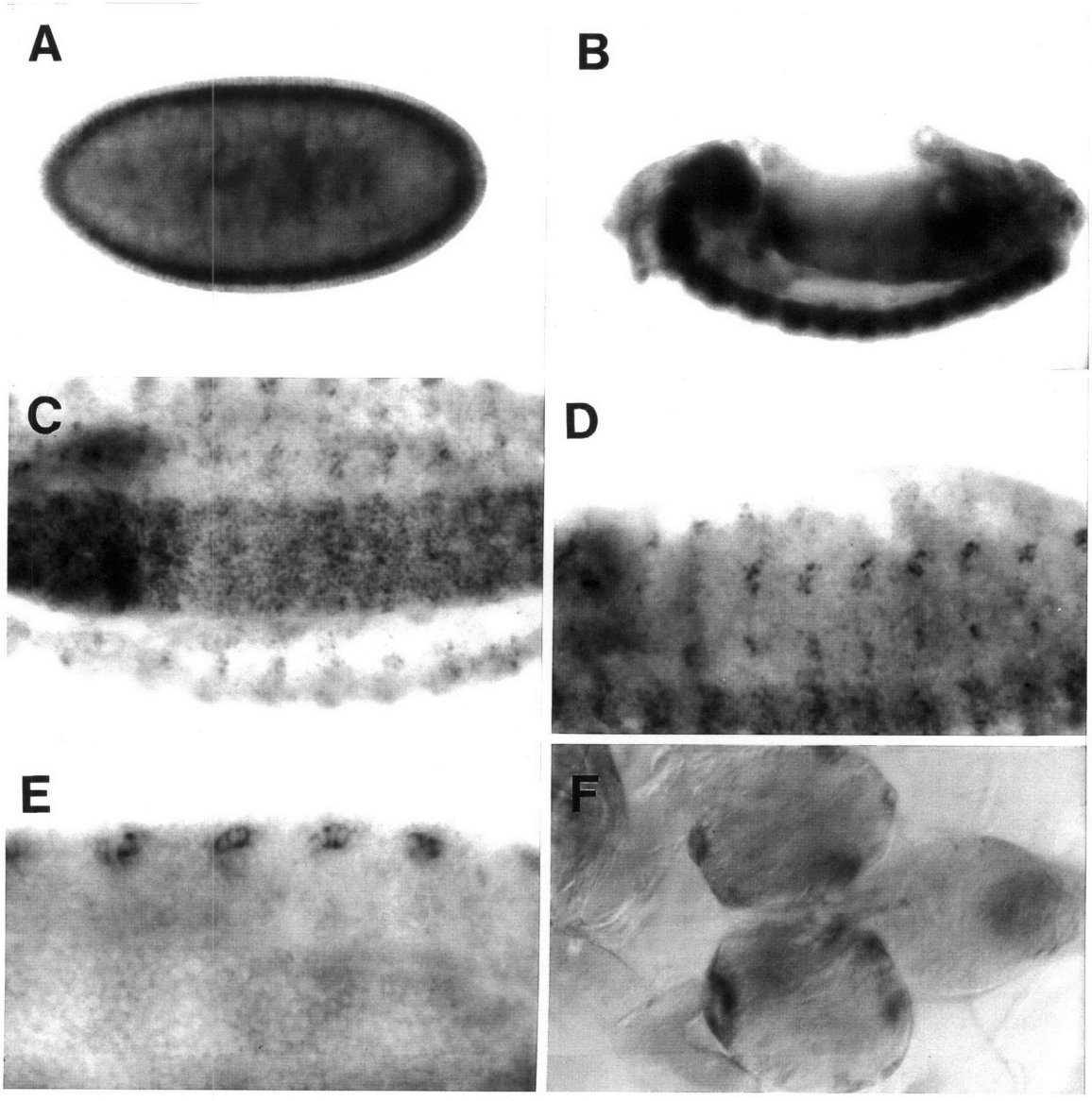


Figure 3. Expression of KLP64D mRNA

The expression pattern of KLP64D is shown via whole mount *in situ* hybridization. Anterior is to the left in all panels. (A) Maternally transcribed KLP64D is seen in embryos at the cellular blastoderm stage. (B-E) In older embryos, KLP64D is expressed in the CNS and PNS. (B,C) Strong expression is seen in the brain and ventral nerve cord. (C-E) KLP64D is expressed in all 4 peripheral sensory neuron clusters within the abdominal segments. (F) In late third instar larvae, expression is seen in the brain hemispheres and ventral nerve cord.


A
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Appendix II: Summary of Third Instar Larval Lineage Analysis.

In order to analyze the relationships between cells within the developing visual system and cells within the proliferation centers in the third instar larval brain, a lineage analysis was carried out using the lineage tracing construct developed by Konrad Basler and Gary Struhl (1993; see chapter 3).

In the initial experiments, mosaic clones were detected via an X-gal stain (see chapter 3). Clones induced during the first instar (25-44 hours AEL, after egg laying; n=165) were compared to those induced during late second/early third instar (67-88 hours AEL; n=21). All clones were analyzed during the late third instar. A summary of clone distribution for these induction times is shown in Table 1.

Following induction in first instar, more than 25% of the brain hemispheres displayed clones which included cells within the outer proliferation center (OPC) and the zone of lamina neurogenesis. These clones have been referred to as "radial clones" (see chapter 3). Over half of the radial clones extended into the developing lamina. A smaller number of hemispheres included clones restricted to the OPC (7%). In contrast, clones extending from the glial precursor zones into the lamina were seen in little over 5% of the brains examined. Approximately 10% of the hemispheres examined displayed clones that included glia in the eye imaginal disc and/or optic stalk, while only 4% showed clones within the subretinal glia. Nearly half of the brain hemispheres were uninformative with regard to lineage in the visual system: of 45% of the hemispheres examined, 32% displayed clones outside of the visual system, 6% contained multiple clones that could not be resolved from each other, and 7% showed no clone at all.

Induction in late second or early third instar animals drastically decreased the number of radial clones (5%). The number of hemispheres with clones restricted to the OPC, however, increased to 21%. A slightly higher percentage of brain hemispheres showed clones extending from the glial precursor zones into the lamina (7%). Roughly the same number of hemispheres contained clones within the eye disc and/or optic stalk glia. There was a substantial increase in the number of hemispheres which displayed clones in the subretinal glial cells (13%). Approximately the same number of hemispheres displayed clones that were uninformative with regard to the developing visual system (44% of hemispheres examined).

Given that clone induction is believed to occur randomly, these data suggest that there are numerous progenitors capable of generating L-neurons, but fewer

progenitors with the capacity to generate L-glia. The proliferation of the L-neuron progenitors appear to decrease substantially in the day preceding the onset of retinal innervation (approximately 94 hours AEL).

Clones within the visual system were analyzed via confocal microscopy. In these experiments, clones were induced within the late first/ early second instar (40-66 hours AEL), or within the late second/early third instar (60-70 hours AEL), and analyzed in late third instar. The brain were labeled with anti-β-galactosidase, to detect the mosaic clone, and with either anti-RK2 (Campbell et. al., 1994), to detect glial nuclei, or MAb44C11 (Bier et. al., 1988 ; Robinow et. al., 1988), to detect neuronal nuclei (see chapter 3). The data from these experiments is summarized in Table 2. It is important to note that the confocal affords a higher level of resolution, and that we were, therefore, often able to analyze several clones within single brains. We have examined the borders of a total of 36 L-neuron clones and 58 L-glial clones. As expected from previous work (Winberg et. al., 1992), none of these clones showed overlap with the other cell type. When the clones were induced between 60-70 hours AEL, a number of glial clones included a small number of cells that were dispersed across the lamina. This data emphasizes that the L-glia appear to truly migrate into the tissue, and are not simply pushed into the lamina anlage by the orientation of their divisions.

In addition to examining lamina clones, we examined clones containing the glia which surround the lamina, namely the medulla and the subretinal glia (see chapter 3). We examined a total of 8 hemispheres which included cells in the medulla glia. All of these clones had a fairly narrow diameter (approximately 5 cells), and each clone included only a small number of cells within the medulla glial layers (see chapter 3, Figure 4B). This indicates that the medulla glia are likely to derive from a large number of progenitors. Clones which included glia in the eye disc, optic stalk and the subretinal glia were examined by both Nomarski optics and confocal microscopy. This data is summarized in table 3, and the number in parentheses represents the number of each type of clone examined via confocal microscopy. Of a total of 87 clones, 30 were restricted to the subretinal glial layer, and 27 were restricted to the eye disc and/or optic stalk. This indicates that there is a divergence in the disc/stalk and subretinal glia lineages. However, 20 clones contained both subretinal glia and glia within the optic stalk, and 10 clones contained all three types of glia. This data indicates that, at some point during larval life, a common precursor cell exists.

This lineage tracing system may be used to address a number of other issues.

(1) Do L-glia within the satellite layer migrate into the tissue ahead of retinal input? As discussed in chapter 3, cells within the "deep" layers of L-glia migrate into the lamina anlage just ahead of retinal input. This experiment was done by examining the expression of an early glial marker, RK2, in innervated and non-innervated brains. Cells within the deep L-glia layers begin to express RK2 independently of retinal input. However, in non-innervated brains, cells within the satellite layer do not express the RK2 antigen. It is possible that this reflects the complete lack of glial migration into the satellite layer in the absence of retinal cues. Alternatively, glia may begin to migrate into the satellite layer, as well as the deep L-glia layers, but may simply not express the RK2 marker. In order to determine which of these possibilities is correct, mosaic clones could be examined in a *sine oculis*^{1} background (Fischbach and Technau, 1984; Cheyette et. al., 1994). The brains could be labeled with anti-β-galactosidase, to detect the clone, anti-HRP, to detect retinal innervation, and with anti-RK2, to easily distinguish between the superficial satellite layer and the deeper layers of L-glia (see chapter 3).

(2) Are cells found within the lamina anlage 24 hours prior to retinal input truly L-glia? One way to answer this question would be to compare the "L-glial" clones in these young brains to the distribution of the RK2 marker. However, it is not known whether RK2 expression is temporally regulated. The immature L-glia may not express the RK2 antigen this early in the animal's life. An alternative experiment could be done, in which, following clone induction during second instar, the animals are pulse labeled in vivo with BrdU during the period of lamina gliogenesis (see chapter 2). The animals would be analyzed during late third instar, and a correlation could be made between the distribution of BrdU + cells and cells within the putative L-glial mosaic clones.

(3) Is there a time period at which the multipotent progenitor of the subretinal glia, glia in the optic stalk, and glia in the eye disc disappears? Our analysis indicates that, of the clones which include these cell types, over half separate the subretinal glia from glia in the optic stalk and eye disc. However, we were able to find examples of clones (n=10) which included all three types of cells. One possibility is that both multipotent and restricted progenitors co-exist. Alternatively, over time, a multipotent progenitor loses its capacity to generate all three cell types. Due to the "background" activity of our hs-FLP construct, we cannot distinguish between these possibilities. This experiment requires a hs-FLP construct that is less sensitive to

temperature variation.

References

Bier, E., Ackerman, L., Barbel, S., Jan, L.Y., and Jan, Y.N. (1988). Identification and characterization of a neuron specific nuclear antigen in *Drosophila*. *Science*. **240:** 913-916.

Campbell, G., Göring, H., Lin, T., Spana, E., Andersson, S., Doe, C.Q., and Tomlinson, A. (1994). RK2, a glial specific homeodomain protein required for embryonic nerve cord condensation and viability in *Drosophila*. *Development*. **120:** 2957-2966.

Cheyette, B.N.R., Green, P.J., Martin, K., Garren, H., Hartenstein, V. and Zipursky, S.L. (1994). The *Drosophila sine oculis* locus encodes a homeodomain containing protein required for the development of the entire visual system. *Neuron*. **12:** 977-996.

Fischbach, K.-F., and Technau, G. (1984). Cell degeneration in the developing optic lobes of the *sine oculis* and *small optic lobes* mutants of *Drosophila melanogaster*. *Devl. Biol.* **104:** 219-239.

Robinow, S., Campos, A.R., Yao, K.M., White, K. (1988). The *elav* gene product of *Drosophila*, required in neurons, has three RNP consensus motifs. *Science*. **242:** 1570-1572.

Struhl, G. and Basler, K. (1993). Organizing activity of wingless protein in *Drosophila*. *Cell*. **115:** 903-911.

Winberg, M.L., Perez, S.E., and Steller, H. (1992). Generation and early differentiation of glial cells in the first optic ganglion of *Drosophila melanogaster*. *Development*. **115:** 903-911.

Table 1: Distribution of mosaic clones induced in first vs. second instar larvae

	% 1IL (n=165)	% 2IL (n=121)
OPC into lamina neurogenesis	11	2.4
OPC into lamina	14.5	2.4
From L-glial precursor zone	5.4	7.4
lamina only	3	5.7
eye disc +/- optic stalk	9.6	9.9
subretinal glia	4.2	13.2
no clone	6.6	14
multiple, not well spaced	6	2.4
OPC only	7.3	21.4
other	32.4	21.2

Table 2: Description of selected clones analyzed via confocal microscopy

	1IL	2IL
L-neurons	24	12
L-glia		
even distribution	6	20
cells dispersed	0	9
differentiation between layers	6	13
clone extends anteriorly	2	2
OPC	4	30
medulla	4	4

Table 3: Summary of clones including subretinal glia and glia within the eye disc and optic stalk

subretinal only	25 (5)
subretinal and optic stalk	13 (7)
optic stalk +/- eye disc	16 (11)
all three glial cell types	6 (4)

Author's note:

Due to the high level of background with the current hs-FLP constructs, we were unable to examine the relationship between cells at the lamina's anterior furrow. Although their movement is coordinated, the lamina furrow is morphologically quite different from the morphogenetic furrow in the eye disc. The eye disc furrow consists of a single row of cells in which the nuclei shift basally. The lamina furrow is 4-5 cells deep. Cells in S phase are seen at two points along the lamina furrow's posterior wall, and at one point along the furrow's anterior wall. It is not clear how the anterior divisions are related to the posterior divisions, nor whether each furrow "daughter" moves entirely around the furrow before entering the lamina.

We had also attempted to analyze mosaic clones in a so^1 background. Because *so* is found on the second chromosome, this experiment requires an insertion of the lineage construct on a chromosome other than 2. We requested an insertion on the 3rd chromosome. In our hands, animals carrying this chromosome never showed *lacZ* expression.