Eye-brain interactions in the development and maintenance of the *Drosophila* visual system

by

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Abstract. The adult visual system of Drosophila melanogaster is composed of the external compound eye and the optic lobes of the brain. Although the size of the compound eye is variable, the size of the underlying ganglia is always perfectly matched. Contact between the developing eye and the first optic ganglion, the lamina, is established when retinal axons first grow across the optic stalk during third instar. Axon ingrowth triggers the final cell divisions of neuroblasts that give rise to lamina neurons, providing an initial mechanism for generating the appropriate numbers of neurons in the lamina. In contrast, we demonstrated that lamina glial precursor cell divisions occur independently of retinal axon ingrowth, thereby demanding a different mechanism. Glial cells were shown to arrest in development prior to being incorporated into the lamina, requiring an inductive signal in order to complete their differentiation. In the absence of this signal, glial cells are eliminated. The mechanistic difference between regulation of numbers of neurons and glia in the lamina led us to examine lineage relationships between these two cell types. Glial cells were shown to derive from a different lineage from that of lamina neurons.

An enhancer trap screen was performed to identify genes involved in lamina development. I selected one strain, named IV27b, for further study because its expression in the lamina was paralleled in the retina, raising the possibility of the coordination of IV27b expression between ganglia. Mutations made at the locus show pre-adult lethality. I performed a mosaic analysis to examine the role of the IV27b locus in the visual system. The major finding in this analysis was that mutant retinal neurons, which appear structurally normal, result in degeneration of target neurons directly underlying the mutant patch. This identifies a previously unrecognized anterograde trophic mechanism. Interestingly, the photoreceptors persist in the absence of their target neurons. Lamina glial cells also require the gene product for survival. Finally, clones of mutant tissue in the brain lead to retrograde degenerative effects. Because defects in this locus reveal the requirement for ongoing communication between cells in the visual system, we have named the locus *incomunicada.*

The *incomunicada* gene was cloned starting from the P insertion. Three mutant alleles show chromosomal breaks within a small interval; this interval was used to probe for cDNAs. Germline transformation with a 12.5-kb fragment encompassing this interval leads to full rescue of lethality and of visual system phenotypes. Sequence of the cDNA and surrounding genomic DNA predicts a novel secreted molecule. Antibodies against the protein show vesicular staining and axonal transport. Therefore, *incomunicada* is presented as a candidate effector molecule for intercellular interactions.

Thesis supervisor: Dr. Hermann Steller
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This work is dedicated to the memory of my mother,
Carolyn Rowe Winberg,
who encouraged me to strive,

and to my family,
Jim, Kathy, Lisa and Eric
because they know and understand,
and go on.

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Chapter I. Introduction

Development can be viewed as a series of steps whose goals are to generate a diversity of cells and to assemble them into a functional system. With regard to development of the nervous system, this may require complex interactions to coordinate cells over long distances. For example, the many neurons in an adult neural circuit are often generated from distinct precursor populations during embryogenesis. In order to perform efficiently, animals must regulate the proportions of different types of neurons that are incorporated into such a circuit in different locations. Likewise, the ratio of neuronal to non-neuronal cells in a local field must be controlled. The strategies used to accomplish these goals may vary between species; even within a particular animal several mechanisms may be employed. Nonetheless, it is likely that the total number of strategies used throughout the animal kingdom is relatively small. Despite their obvious differences, a number of similar molecular and cellular mechanisms have been noted in the development of vertebrate and invertebrate nervous systems (McKay, 1989; Goodman and Shatz, 1993). Lessons from one experimental system may be extrapolated to another.

One theme in the development of the nervous system is the importance of cell contacts. Direct contact between cells can affect neuronal and non-neuronal fate specification among seemingly identical cells (Eisen, 1992; Greenwald and Rubin, 1992), patterns of cell migration (Klämbt et al., 1991; Hatten, 1993), and temporal and spatial control of axon outgrowth (Bentley and Caudy, 1983; Sretavan, 1993; Wizenmann et al., 1993). Cell contacts can also help regulate the abundance of different cell types through either proliferative (cell cycle) or degradative (cell death) control (see below). Cells can also influence one another's behavior though soluble signals (Jessell and Melton, 1992; Goodman and Shatz, 1993; Pini, 1993), or through molecules deposited in the extracellular matrix (Jackson et al., 1991; Hynes and Lander, 1992). In this way, intercellular signals may pass over long distances, or persist for a long time after the signalling cell is removed. This thesis focuses on the role of intercellular interactions in controlling cellular differentiation and maintenance within the developing and adult nervous system. Principally, I have examined the role of afferent neurons in regulating the development of target tissues and in maintaining these cells in the adult animal.

Experiments in this thesis have used the adult visual system of *Drosophila melanogaster* as a model for studying cellular and molecular interactions in development. The visual system is highly amenable to molecular

and genetic study: it is composed of a small number of cell types, and is organized into repeated arrays whose structure is well-described (Trujillo-Cenoz, 1965; Strausfeld, 1976; Fischbach and Dittrich, 1989). Detailed structural knowledge is especially helpful in the analysis of genetic mosaics (Meyerowitz and Kankel, 1978; Thaker and Kankel, 1992; Xu and Rubin, 1993). Markers for specific cell types within the visual system are increasingly available, facilitating histological analysis of developmental mutants (for example, Zipursky et al., 1984; O'Kane and Gehring, 1987; Buchner et al., 1988; Bellen et al., 1989; Bier et al., 1989; Wilson et al., 1989; Mlodzik and Hiromi, 1992). In addition to structural analysis, electrophysiological and behavioral assays can be used to screen for new mutations, and to test visual function in different genetic backgrounds (Hotta and Benzer, 1969; Pak et al., 1969; Fischbach and Heisenberg, 1981). Visual phenotypes span the range from minor to severe. Even gross aberrations can be tolerated because, under laboratory conditions, the visual system is dispensable for viability and stock maintenance. Finally, as is the case for all *Drosophila* studies, advances in molecular genetics and gene transfer technology provide rapid access to the genes responsible for mutant phenotypes, and enable the direct manipulation of selected genetic loci (for example Rubin and Spradling, 1982; Karess and Rubin, 1984; Steller and Pirrotta, 1986; Ashburner, 1991). I have used a variety of genetic and cell biological tools to study the generation of particular glial cells, and to characterize a gene required for cell survival in the visual system.

In this introductory chapter, a description of wild-type visual system development and adult structure will be presented. This will be followed by short considerations of the role of glial cells in nervous development and function, and of mechanisms regulating the number of cells in the mature nervous system. Finally, a brief survey of mutations affecting the *Drosophila* visual system will be given.

The *Drosophila* adult visual system

The compound eyes of *Drosophila* are the most obvious feature of the adult head. Their large size reflects their importance to the animal: the eye provides the fly with its primary sensory input, and has a profound impact on its behavior (for example, Heisenberg and Buchner, 1977). The eyes are positioned directly over the central nervous system components of the visual

system, the optic lobes of the brain. The optic lobes are subdivided into four ganglia; from lateral to medial these are the lamina, the medulla, the lobula and the lobula plate. Together the optic ganglia comprise over half of the total brain volume, again indicating the importance of visual information to the fly.

An important aspect of the visual system is its organization into reiterated units. At a gross level this is seen in the parallel arrangement of the different ganglia into cortex (cell body) and neuropil (synaptic zone) regions (Figure 1.1). Looking more closely one sees that each ganglion is subdivided into columnar units, organized into parallel two-dimensional arrays. This is most obvious in the eye. Retinal cells are arranged into ommatidial units, each consisting of eight neurons and twelve non-neuronal cells topped with a lens (Figure 1.2). The cells within an ommatidium are organized in a highly reproducible pattern, permitting unambiguous identification of each cell and cell type. The lamina, or first optic ganglion, also contains an array of columnar elements; in this tissue the repeated unit is called a cartridge. Each cartridge has five neurons, whose cell soma adopt a stereotypical arrangement. Cartridges are separated from each other, and from neighboring tissues, by specialized glial cells (Figure 1.3). The medulla and lobula have neurons arranged in columnar units as well, though this organization is partially obscured by the increasingly prominent horizontal or tangential elements (Fischbach and Dittrich, 1989). Generally, columnar axonal fibers project medially from each cortex into the neuropil region of the next ganglion. In each case, projections are made in strict retinotopic order, such that a two-dimensional representation of the visual field is precisely reproduced at each level of processing (though it is inverted at each chiasm) (Meinertzhagen, 1973; reviewed in Kunes and Steller, 1993). This stereotypic arrangement, together with the large degree of reiteration, has permitted detailed structural examination of single units to be extrapolated over whole ganglia. It has also aided in studies that perturb structure through genetic or physical manipulations.

Each ommatidium contains three types of retinal neurons; these are distinguishable by their spectral sensitivities and by the level of their projections. The outer photoreceptors, R1-6, project to the lamina. In tangential sections they show large-diameter rhabdomeres, containing a rhodopsin that is sensitive to visible light. In contrast the inner photoreceptors, R7 and 8, have small-diameter rhabdomeres. The inner rhabdomeres are stacked within an ommatidium, such that only one at a time can be seen in tangential sections

(Figure 1.2). The projections of the UV-sensitive R7 and the blue-light-sensitive R8 terminate at different depths in the medulla. Ommatidia also contain a mechanosensory bristle cell, secretory cone cells, and pigment cells, whose colored granules serve to optically isolate one ommatidium from its neighbors (Ready et al., 1976).

The five lamina neurons within a single cartridge represent five different cell types, though they are often mistakenly considered as a single class. The five types can be distinguished by the stereotyped positions of their cell soma and by differential terminations of their axons within the medulla neuropil (Fischbach and Dittrich, 1989). Thus the different types of lamina neurons are connected with distinct columnar and tangential elements in the medulla, thereby making unique contributions to visual information processing. Different types of lamina neurons also display specialized synaptic connections with photoreceptor neurons. The pattern of synaptic connections within the lamina neuropil has been worked out in detail (Meinertzhagen and O'Neil, 1991). It is characteristic of this system that afferent fibers form synapses directly on the axons of their neuronal targets, which lack dendritic structures. Only two types of lamina neurons, L1 and L2, receive direct phototransduction signals from the retina, although all types of lamina neurons are synaptically coupled to each other. The other lamina neurons contribute feedback synapses to modulate retinal activity, and numerous lateral projections (Meinertzhagen and O'Neil, 1991). As a result, individual axons contain both presynaptic and postsynaptic specializations, often in near proximity to one another.

Although retinal projections show point-to-point specificity, in the mature lamina, axons from a single ommatidium separate to innervate six different cartridges. (The fibers of R7 and R8 pass through the lamina without making any connections.) Consequently, a single cartridge receives input from six clustered ommatidia in a precise retinotopic pattern. This interweaving is the basis of neuronal superposition, the first step in the convergence of visual information from different ommatidia (Trujillo-Cenoz, 1965; Braitenberg, 1967). In addition to forming synapses, axons in the lamina neuropil also participate in specialized junctions with glial cells (Saint Marie and Carlson, 1983b; Stark and Carlson, 1986). The importance of these neuron-glia junctions is not known, but many authors have suggested that they permit mobilization of nutritients from the glial cells to meet neuronal metabolic demands. Lamina glial cells form gap junctions and septate junctions with one another in a tissue-

wide network. [Septate junctions, homologous to vertebrate tight junctions, form the blood-brain barrier in insects (Treherne, 1985).] This simultaneously provides electrical isolation for individual neural circuits, and cellular continuity across the lamina, possibly facilitating transport of small molecules to and from neurons in the interior of the tissue.

Development of the *Drosophila* visual system

The eye and the optic lobes of *Drosophila* arise from separate precursor populations set aside in the embryo. These two populations remain physically apart through most of the larval period. It is therefore convenient to consider events in optic lobe development separately from events in the eye disc.

Retinal development in larval and pupal stages

The eye disc develops as a simple epithelium, expanding from a few cells in the embryo to a few thousand cells by the middle of the third larval instar stage (Garcia-Bellido and Merriam, 1969). An early hallmark of cellular differentiation in eye discs of third instar larvae is the appearance of the morphogenetic furrow, a dorsal-ventral indentation that sweeps anteriorly across the eye disc from the optic stalk (Ready et al., 1976). Anterior to the furrow, cells appear unpatterned in histologic preparations (though there certainly is differential gene expression in this region, see Heberlein et al., 1993; Ma et al., 1993). Posterior to the furrow, cells begin to organize into clusters, with characteristic spacing between each cluster along the dorsal-ventral axis (reviewed in Tomlinson, 1988). Each cluster grows by successively recruiting neighboring cells according to a stereotyped temporal and spatial pattern. The first eight cells resident in the cluster correspond to the retinal neurons. (Clusters initially include a transient member, the "mystery cell", that is not represented in the adult eye.) Other cells in the retinal field may later be recruited as pigment, cone or bristle cells, or they may be eliminated by cell death (Cagan and Ready, 1989a, b; Wolff and Ready, 1991). During the late larval and early pupal stages, steps in cellular differentiation proceed from posterior to anterior, in delayed correspondence to the morphogenetic furrow. Scanning along the anterior-posterior axis during these stages gives the equivalent of a developmental time course, with the oldest cells at the posterior

border of the disc (Figure 1.4). Ommatidial maturation is accompanied by characteristic morphological changes, and can also be visualized by the sequential expression of differentiation antigens from the posterior to increasingly anterior positions (Jan and Jan, 1982; Zipursky et al., 1984). Partway through pupal development the visual field loses this anterior-posterior temporal polarity, with later events following different spatial rules. For example, secretion of the lenses occurs uniformly across the eye, whereas pigment deposition begins at the circumference, proceeding radially inward (Ashburner, 1989; Cagan and Ready, 1989a).

A large number of mutations have been generated that disrupt cell patterning and cell fate determination in the *Drosophila* retina. (For reviews see Tomlinson, 1988; Ready, 1989; Rubin, 1989; Banerjee and Zipursky, 1990; Hafen and Basler, 1991; Greenwald and Rubin, 1992). Analysis of these mutants has confirmed that fates are assigned by a series of cell-cell interactions, rather than through lineage restrictions. Thus, the cells anterior to the morphogenetic furrow are equipotent. At the furrow, clusters begin to form at regular intervals. The spacing between clusters is determined, not by any lineage mechanism, but by a process of lateral inhibition, mediated in part by the activities of *scabrous*, *Notch* and the *Drosophila* EGF receptor homolog (Baker and Rubin, 1989; Cagan and Ready, 1989b; Baker et al., 1990). Within the cluster, R8 is specified first; subsequent neuronal fates are specified in a series of contact-dependent inductive steps. Likewise, contacts mediate the induction of cone and pigment cells, and the elimination of extra interommatidial cells (Cagan and Ready, 1989a; Wolff and Ready, 1991). Failure of these induction events may lead to a scarred or roughened eye. Although fates are acquired asynchronously and in some cases depend on unique identified molecular interactions (such *sevenless* and its bride determining R7 neurons, Krämer et al., 1991), it appears that many cellular decisions are made using different combinations of a relatively small number of shared molecules. The *Drosophila Ras1* gene plays a role in ommatidial spacing, as well as the sequential determination of various photoreceptor neurons (Simon et al., 1991; Heberlein et al., 1993). The *Notch* protein also participates in a series of fate-restricting steps in retinal development (Cagan and Ready, 1989b). It appears that ubiquitous cellular activators, such as *ras*, may cooperate with unique fate specifiers, many of which are as yet unidentified, to produce the variety of cell types in the *Drosophila* retina (Feig, 1993).

Development of the optic lobes

The cellular events in insect optic lobe development have been examined by Nordlander and Edwards (1969), White and Kankel (1978) and Hofbauer and Campos-Ortega (1990). Historically, special attention to the development of the retina-lamina complex has been given by Meinertzhagen (1973), Trujillo-Cenoz and Melamed (1973) and Macagno (1979). These studies have complemented and reinforced each other; unless otherwise indicated, they are the basis for statements made in the following paragraphs.

The optic lobe primordia are derived from a group of cells that invaginate from the dorsal embryonic ectoderm, migrating ventrally to merge with the central brain in a ventrolateral position (Green et al., 1993). Cells in this region become morphologically identifiable as neuroblasts during larval development. The neuroblasts separate into two zones of dividing cells, called the inner and outer proliferation centers. The inner proliferation center is shaped like a rod, positioned along the medial-lateral axis. It contains precursor cells that give rise to neurons and glia of the lobula plate, the lobula and the inner medulla. The outer proliferation center, initially shaped like a ribbon wrapped around the inner rod, gives rise to cells of the outer medulla and lamina. Stem cell proliferation causes these centers to expand and separate from one another. This growth is later accompanied by production of ganglion cells, shed from the margins of the proliferation centers into the space between them. Late in the third instar stage, the still-dividing blast cells of the proliferation centers have become separated by a large number of mitotically quiescent cells (Figure 1.5). Many of these are already differentiating as neurons and glia of the inner ganglia.

Also during third instar, retinal axons begin to grow from the eye disc across the optic stalk, an epithelial tube connecting the eye disc to the brain. At the base of the stalk they enter the presumptive lamina, residing at the lateral margin of the optic lobe. Reflecting the posterior-to-anterior progression of events in the retina, axons arrive in the brain beginning at the posterior edge of the lamina. Axons arriving later assume increasingly anterior positions. During this phase, the lamina is crescent-shaped, curved in parallel to the outer proliferation center, from which it is derived. The ingrowing retinal axons spread out along the front of the crescent, representing the dorsoventral axis.

Recent work has shown that retinal axons can navigate independently of one another to find their correct retinotopic targets, suggesting that axons are sensitive to positional cues along this axis (Kunes et al., 1993). Axons arriving at the anterior edge pass by a narrow, crescent-shaped zone of mitotically active cells, called the lamina precursor cells. This close juxtaposition is not merely coincidence: retinal fibers induce mitosis in these precursor cells, giving rise to lamina neurons (Selleck and Steller, 1991). After spreading along the crescent front, photoreceptor axons turn medially and project into the brain, with R7 and R8 extending into the medulla neuropil region and the other axons terminating in the presumptive lamina. Glial cells in the target area are positioned where retinal growth cones make each of these turns and stops, leading to the suggestion that glia may provide some kind of positional information.

During the pupal stage, cellular differentiation continues, culminating with the formation of synapses between various neurons. In addition, a number of morphogenetic events take place. Head eversion and the expansion of the optic stalk bring the lamina directly under the retina. The medulla rotates into position, with the result that projections from anterior neurons of the retina and lamina terminate in the posterior region of the medulla; in horizontal sections crossing axons are seen in the chiasm between the lamina and medulla. Additionally, the lobula moves from a lateral to a medial position, inserting between the medulla and the central brain and forming the inner chiasm. Though these events have been described for wild-type animals, the mechanism by which they are achieved is not understood.

Neuronal cell birth, structure and function in the visual system has been studied at length. Mostly for technical reasons, however, few details of *Drosophila* glial cell biology have been elucidated. Landmark studies in adult insect glial cell structure have raised important functional and developmental questions (e.g. St. Marie and Carlson, 1983), and in some cases have provided hypotheses as to why glia are typically so complex, with numerous specialized membrane structures and processes (e.g. Stark and Carlson, 1986). Additionally, investigators examining the cellular assembly of the lamina have noted that glial cells reside in proximity to key choice points in axonal navigation (e.g. Trujillo-Cenoz and Melamed, 1973). This has led to the proposal that glia are involved in photoreceptor axon guidance, an interesting idea which is as yet without experimental support. Because work in the next

chapter focuses on glial cells, the following section provides a short review of glial cell generation and function, drawing from *in vitro* and *in situ* studies in *Drosophila* and other species.

Glial cells in the development of the nervous system

The word *neuroglia*, or *nerve glue*, was first used by the German pathologist Virchow in 1856 (Somjen, 1988). It names the first function to be ascribed to vertebrate glial cells: they were viewed as a matrix to hold neuronal architectures in place. Since then, many investigators have looked at the interactions between different types of glia and neurons in culture and during development. These studies have implicated glia in numerous additional functions, including guidance roles in neuronal migration and axon outgrowth, secretion of growth and trophic factors, modulation of neurotransmission, and maintenance of homeostatic pH and ionic conditions. This section describes some of what is known of glial cell origins and functions, comparing vertebrate and invertebrate models.

Gliogenesis

Glial cell origins have been precisely determined in only a few cases. Glia generally resemble neurons in being ectodermal derivatives. Lineage analysis of vertebrate glial cells has been performed by dye labeling and retroviral infection. Depending on the experimental system, labeled clones consisting only of glial cells may be found, suggesting the existence of a unipotential precursor (Bronner-Fraser and Fraser, 1989; Hall and Landis, 1991). In contrast, glia may be labeled along with neurons, indicating a multipotential precursor cell type (Wetts et al., 1989; Frank and Sanes, 1991). At present it appears that neural crest derivatives probably all arise from a single stem cell type (Stemple and Anderson, 1992). Reminiscent of hematopoeitic cells, this stem cell would give rise, in different branches of a family tree, to precursors that become progressively restricted to produce neuronal or glial progeny. In some cases the restriction may occur late in the lineage, allowing small clones with a mixture of cell types. In other cases, an early restriction would give rise to homogenous clones. There may be a corresponding single precursor type from which all central neurons and glia

would be derived (Frederiksen et al., 1988, Cunningham, Renfranz and McKay). It remains to be worked out when various precursor cells become restricted, and by what mechanism.

In the central nervous system of *Drosophila* embryos, lineage tracing has permitted the identification of different types of precursor cells in the ventral neurogenic region. Cells that delaminate from the neuroectoderm have generally been called *neuroblasts*, based on cell morphology (large, round and easily stained by specific dyes) rather than on identification of their progeny. However, one well-studied CNS "neuroblast" lineage gives rise to glial cells as well as neurons positioned at the midline (Rothberg et al., 1988; Jacobs and Goodman, 1989b). The midline precursors derive from the border of the mesoderm and the ectoderm; due to their unique position it was not clear that their multipotency would be a general feature of embryonic neural precursors. The recent identification of an additional multipotential CNS precursor, which is *not* in the midline, has raised that possibility (Udolph et al., 1993). Nevertheless, at least some of the cells in the central nervous system derive from precursors with a restricted potential. In particular, the longitudinal glia arise from glioblasts that form at the lateral margin of the neurogenic field. The glioblasts morphologically resemble neuroblasts, but do not produce neurons. Interestingly, these cells are responsive to mutations in the neurogenic genes (see below), suggesting that they may be selected out of the neuroectoderm via a similar molecular interaction (Jacobs et al., 1989a). Because all of these precursors are selected out of a field of equipotent cells (see below), it is unlikely that they are constrained by any lineage restrictions prior to the time of "neuroblast" delamination, but rather respond to positional information along the dorsoventral axis. Therefore the *Drosophila* CNS is generated from at least three, and possibly more, distinct precursor cell types. These cell types are distinguishable from each other as early as they can be distinguished from non-neural ectodermal cells.

In contrast to the CNS, cells in the *Drosophila* peripheral nervous system, such as the external sense organs, are not generated in the ventral neurogenic region. Rather, PNS structures are derived from precursor cells that are selected from the embryonic peripheral ectoderm (see below). These precursors are multipotent: each divides to generate all the cells of the sense organ, including sensory neurons and glia-like support cells (Bodmer et al., 1989). Likewise the retina, which in flies is part of the peripheral nervous

system, contains multipotent precursor cells able to produce neuronal and non-neuronal progeny (Ready et al., 1976; Lawrence and Green, 1979). To date, no example of a peripheral glioblast has been reported in *Drosophila*.

Glia as patterning elements

Certain classes of glia have been implicated in generating the pattern for neuronal migration and axon outgrowth. A well-known example is in the developing vertebrate CNS: neurons migrating from the proliferation zones follow pathways laid out by radial glia. It has been proposed that particular migratory pathways may participate in establishing neuronal phenotypes as well as directing neurons to their destinations (reviewed in Hatten, 1993). Another example is seen in the development of the corpus callosum. Axons from one side of the brain project to their contralateral targets across a "glial sling." Surgical disruption of the sling prevents axons from growing across. The sling can be functionally substituted by a nitrocellulose bridge only if the nitrocellulose is innoculated with immature astrocytes, suggesting that these glia play an essential, or at least permissive, role for the projection of central axons (Silver et al., 1982). Axons in the optic nerve also extend growth cones along a glial substrate; growth cone guidance in this case can be disturbed by antibody blockade of specific antigens (such as N-CAM: see Silver and Rutishauser, 1984). As a final example, olfactory axons of insects (Oland et al., 1990) and vertebrates (Steindler et al., 1990) are guided to their targets by interactions with glia.

In *Drosophila*, a scaffold of glial cells prefigures the major axon tracts of the CNS (Jacobs and Goodman, 1989b). The overall patterning of the CNS is disrupted by mutations that prevent glial cells (as well as other cells at the midline) from forming correctly (Rothberg et al., 1988; Thomas et al., 1988). In the visual system, glial cells are present along the routes axons must take in the retina, the optic stalk, the lamina, the optic chiasm, and probably elsewhere. These positions have led several authors to speculate that visual system glia may serve guideposting functions (Trujillo-Cenoz and Melamed, 1973; Cagan and Ready, 1989a; Poeck et al., 1993; Shea et al., 1994). *Drosophila* visual axon-guidance mutations specifically affecting glial cells have not been reported, however.

The role of glial cells in neuronal function

While it was assumed early that glia would serve as insulators for excitable cells, more recent studies have increasingly demonstrated that glia make additional important contributions in optimizing neuronal function. Certainly axons of many neurons are sheathed in glial membranes, separating individual axons from their neighbors in a nerve bundle. In vertebrates, in addition to providing insulation, myelin proteins in Schwann cells and some oligodendrocytes actually speed the propagation of action potentials. This enables fast reflexes, in spite of the requirement to pass information long distances. Glia also envelop synaptic sites, where they take up excess neurotransmitter, and re-establish appropriate ionic conditions around the neuron (Henn and Hamberger, 1971). This provides several benefits. First, quick restoration of the membrane resting potential permits inactivation of voltage-sensitive channels; this in turn allows the circuit to fire again. Second, optimal function of the synapse depends on rapidly dampening one signal before the next one arrives, so as to permit discrete repetitive signalling events. Third, some neurotransmitters or their components can be recycled to the presynaptic neuron, reducing the metabolic load. Fourth, in the case of toxic neurotransmitters, glial uptake leads to their neutralization and prevents excitotoxicity (Choi, 1988). Interference with any of these functions, or failure of glial cells to wrap neurons and their synapses, can lead to severe consequences. One illustration comes from the *drop-dead* mutation in *Drosophila*, which affects glial morphology: stunted processes fail to wrap completely around their neuronal neighbors (Buchanan and Benzer, 1993). Flies with this mutation show massive and rapid brain degeneration and early death. It is interesting to speculate that brain degeneration in these mutants may be due to "naked" synapses and excitotoxicity.

Neurotransmitters may also directly affect glial activities. It was long believed that glial cells were "non-excitable" components of the nervous system, but all glia that have been tested so far are electrically active in their own right (see Gilbert et al., 1984; Bormann and Kettenmann, 1988). This potentially provides a method for glia to detect and respond to neuronal activity (Orkand et al., 1966). For example, photostimulation of the honeybee retina causes cAMP production and glycogen breakdown in glial cells, mobilizing an energy source that is subsequently transferred to the neurons (Tsacopoulos et

al., 1988; Coles et al., 1989). In addition, many glia activate ion channels in response to neurotransmitters (MacVicar et al., 1989). Electrical activity of cultured astrocytes initiates waves of calcium flux between cells coupled by gap junctions (Cornell-Bell et al., 1990a). Perhaps these responses contribute to maintaining ionic homeostasis of synaptic regions: the excess ion load is spread to gap-junctionally coupled cells at a distance, presumably in a synaptically quiescent area (known as the spatial buffering hypothesis, Bormann and Kettenmann, 1988; Karwoski et al., 1989). Another hypothesis is that glial neurotransmitter receptors mediate glial maturation at synaptic sites during development. Cultured glia respond to neurotransmitters by exiting from the cell cycle and initiating process formation. Presumably the processes are intended to wrap around the (neuronal) source of the transmitter (Condorelli et al., 1989; Cornell-Bell et al., 1990b). This achieves both the appropriate insulation of the synaptic site, and the incorporation of the correct number of astrocytes into the neural circuit.

Glia secrete growth and trophic factors

A wide variety of factors synthesized by glial cells promote neuronal survival and/or neurite outgrowth in culture. The list of such molecules derived from gliomas, glial cell lines or primary glial cells includes the neurotrophins (NTs), ciliary neurotrophic factor (CNTF), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), glial-derived neurotrophic factor (GDNF), and the insulin-like growth factors (IGF-1 and IGF-2). As is apparent from the names given to them, many of these factors are not exclusively derived from glia, nor exclusively directed at neurons. Some of these factors are produced by one subset of glia and have mitogenic or trophic effects on other glial types (for example Richardson et al., 1988).

Among these soluble factors, greatest historical attention has been paid to nerve growth factor (NGF), the prototypical neurotrophin. The growth-promoting properties of NGF were first observed following implant of a particular sarcoma cell type into mice. The grafts elicited massive invasion by fibers of local sensory and sympathetic neurons, as well as hypertrophy of sympathetic ganglia elsewhere in the experimental animals. These results suggested that the tumor was secreting a diffusible agent that had growth-altering effects on specific types of neurons (for review see Levi-Montalcini, 1987). Subsequent

studies demonstrated that the agent, NGF, was essential for normal development of sensory neurons, and that this requirement reflected the role of NGF in promoting neuronal survival (Barde, 1989).

Biologically active NGF is synthesized in the target tissues of sympathetic nerves, sensory neurons and cholinergic neurons of the forebrain. It is produced in limiting amounts, supporting the hypothesis that ingrowing fibers must compete for available factor (Barde, 1989). NGF preproprotein is found in a variety of other cell types, but in many of these cases it is processed into a biologically inactive form (Edwards et al., 1988a, b). The secreted neurotrophic factor is taken up by high affinity receptors in nerve terminals and retrogradely transported. In addition to its classical neurotrophic activity, NGF may play other roles in peripheral nervous development (for example Lee et al., 1992).

NGF is the founding member of a family of "neurotrophins," including the brain-derived neurotrophic factor (Leibrock et al., 1989), NT-3 (Enfors et al., 1990; Hohn et al., 1990; Jones and Reichardt, 1990; Maisonpierre et al., 1990; Rosenthal et al., 1990), NT-4 (Hallböök et al., 1991) and NT-5 (Berkemeier et al, 1991). These molecules exert trophic effects on overlapping but non-identical sets of neurons.

In contrast to the restricted specificity of the neurotrophins, bFGF affects a wide variety of cells in different parts of the brain, as well as non-neuronal cells elsewhere. In addition to promoting neuronal survival, it elicits neurite outgrowth in culture assays and acts as a potent mitogen for CNS glia. bFGF also protects neurons from experimentally-induced lesions (for example, Mattson et al., 1989; Cheng and Mattson, 1991). Because bFGF is abundant in the brain, some authors have suggested that it is not a trophic factor in the same sense as NGF: clearly it is not produced in limiting amounts. However, the binding of FGF to its various receptors is mediated by heparan sulfate chains in the extracellular matrix (Jackson et al., 1991; Givol and Yayon, 1992). This raises the possibility that the appropriate matrix components are the real limiting reagent, or that other kinds of molecules in the matrix sequester bFGF in some way. Perhaps some of the FGF in the brain is not constitutively available, but exists as a reservoir to enable rapid response to brain insults (Barde, 1989). Alternatively, trophic effects may be mediated by only a subset of FGF receptors, which may then be the limiting factor (Givol and Yayon, 1992).

The remaining secreted factors in the list are not structurally similar. CNTF was isolated on the basis of its effect on chick ciliary ganglion cells; it has

a novel sequence (Lin et al., 1989). GDNF, which enhances the survival of dopaminergic neurons of the midbrain, is apparently related to transforming growth factor-β (Lin et al., 1993). PDGF is both neurotrophic and mitogenic for glia. It is implicated in glioma formation and has homology to the v-*sis* oncogene (Doolittle et al., 1983; Richardson et al., 1988). As an interesting aside, there are many reports that cultured brain glia produce cytokines such as interleukin-I and tumor necrosis factor-α. However, a more likely hypothesis is that these factors are found in the supernatant of primary astrocyte cultures due to the activity of a contaminating cell type known as microglia. These cells are not glia at all, but rather are derived from lymphoid cells, the classic source of cytokine molecules (Giulian, 1992).

Most of what is known about neurotrophic factors and their receptors has resulted from work in vertebrate systems. However, both mutational approaches and homology-based molecular screens have demonstrated related proteins in *Drosophila*. The best-known of these may be the *sevenless* receptor tyrosine kinase, which is required for induction of the R7 cell fate in the eye (Banerjee et al., 1987; Hafen et al., 1987). While the *sevenless* protein does not specifically act as a neurotrophin receptor, the possibility remains that such a molecule will be found. Mutations in two different EGF-like genes affect embryonic neuro- and gliogenesis at the ventral midline, leading to CNS collapse (Rothberg et al., 1988; Thomas et al., 1988). Different alleles of the *Drosophila* EGF receptor affect embryonic polarity (Price et al., 1989), zygotic morphogenesis, including CNS phenotypes (Schejter and Shilo, 1989), and pattern formation in the compound eye (Baker and Rubin, 1989). Mutations in the gene encoding an FGF receptor homolog prevent specific cells in the nervous system and elsewhere from migrating correctly (Klämbt et al., 1992). NGF-homologous activity has been found in embryos (Hayashi et al., 1992), and genes whose sequences are similar to the high affinity NGF receptor have been identified (Pulido et al., 1992; Wilson et al., 1993). Ligands for these receptors have not been found, though if the vertebrate parallels continue, it is likely that many of them will be provided by glial cells, among others.

One likely example of a glial-derived factor in *Drosophila* is the product of the *anachronism* gene. Mutations at this locus perturb glial function in the optic lobe, resulting in aberrant neurogenesis (Ebens et al., 1993). This in turn leads to severe abnormalities in adult visual system structure. The mutant protein does not obviously resemble known glial proteins, but it is interesting to

compare its effects with the trophic factors listed above. In addition to trophic functions, a number of these molecules have mitogenic properties under certain conditions. This suggests that in addition to influencing the survival of differentiated neurons, glial factors may play an important role in regulating neurogenesis (see below).

Control of cell numbers

Appropriate development of the nervous system requires coordination of the disposition and differentiation of large numbers of cells and cell types. This raises three principle questions. First, how are different types of cells in the nervous system generated? Second, how are the numbers and proportions of different cell types regulated? Third, how are connections between cells established, particularly in the case of neurons that are born at a distance from their synaptic partners? The answers to these three questions are intertwined, and one issue can not be satisfactorily understood in ignorance of the others. Nevertheless, it is possible to isolate specific aspects of each question for theoretical and experimental consideration. This section addresses mechanisms for regulating cell numbers, with particular attention paid to studies of insect systems. Though invertebrate nervous development has been called invariant, sufficient examples of variability have been described as to make insects useful for tackling this problem. Both proliferative and degenerative regulatory mechanisms have been noted in insect nervous system development, and examples of each will be presented and discussed.

Variation in total neuron number

The number of neurons in adult members of the same species may vary widely. In wild populations, variation must depend in part on genetic factors, however analysis of isogenic experimental animals clearly indicates a role for epigenetic determinants (Goodman, 1979; Macagno, 1980). Variation is most evident within populations of functionally similar neurons organized in parallel. For example, absolute size of the primate neocortex varies tremendously (van Essen et al., 1984, 1986; Williams and Rakic, 1986). Also, within a single animal, left-right differences in the size of the visual cortex can be pronounced (Williams and Herrup, 1988). Likewise, the compound eye of insects, which

consists of reiterated units, shows size variability within species, even in isogenic populations (Power, 1943). In a few cases, experimentally-induced differences in neuron number have been correlated with differences in functional effectiveness: newts with fewer CNS neurons require more training to learn a maze (Fankhauser et al., 1955; Vernon and Butsch, 1957), while cats with increased input to the visual cortex apparently have increased visual acuity. The ideal number of neurons may represent a balance between performance benefits and metabolic constraints. In the nematode, a 20% increase in the number of neurons is associated with a 15-30% decrease in fecundity (Ellis and Horvitz, 1986). In *Drosophila*, the number of cells in the adult eye depends largely on the nutritional history of the larva (Power, 1943).

Sources of variation

Variation in the number of neurons in an adult could arise in several ways. Among these are (1) differential acquisition of neural stem cell fates, (2) different production capacities of various stem cells, and (3) variability in the elimination of perceived excess cells. These processes may be regulated through cell-autonomous or non-cell-autonomous mechanisms.

Commitment to stem cell fate

In a few experimental systems, neurogenesis proceeds according to stereotyped patterns of cell division. Such is the case for segmental ganglia in the leech (Macagno, 1980) and the nematode *C. elegans* (Sulston and Horvitz, 1977). These animals are ideal for studying the role of lineage in determining stem cell fates, using genetics and cell ablation analysis. For example, ablating an identified neuroblast in the leech does not affect patterns of cell division of neighboring cells or of the contralateral homologous neuroblast, i.e., no additional cells are generated to make up the difference. This argues that even though surrounding cells are morphologically undifferentiated at the time of ablation, they are incompetent for recruitment into a neuroblast fate, presumably due to lineage restriction. Nevertheless, postmitotic neurons from the contralateral side may cross over to effect phenotypic rescue, indicating recognition of neuronal deficit (Shankland and Weisblat, 1984). Studies in the nematode suggest that most cells are constrained by lineage to a single

possible fate (Sulston and Horvitz, 1977). Some exceptions are neuroblasts that use cell-cell interactions to choose between two or three alternative fates, but even these are restricted by lineage (Sternberg and Horvitz, 1991).

In contrast, neuroblast selection in the *Drosophila* embryo typically operates by a lineage-independent mechanism. The ventral neurogenic region of the CNS begins as a simple epithelium, from which approximately one-quarter of cells are selected as neuroblasts. Neuroblasts are shed medially; cells remaining at the lateral margin are designated epidermoblasts. Laser ablation of delaminated neuroblasts permits "epidermoblasts" to delaminate and take up the neural fate. This indicates that cell fates in the neurogenic region are not fixed by lineage (Doe and Goodman, 1985b). Several mutations affecting the proportions of neural and epidermal cells have been isolated (Garcia-Bellido and Santamaria, 1978; Lehmann et al., 1981; Lehmann et al., 1983). Work of many investigators has indicated that initial specification occurs via the activities of the "proneural" genes, which promote neuroblast formation, and "neurogenic" genes, which prevent epidermoblasts from adopting neural fates. (Molecular and genetic analysis of these genes has been reviewed by Campos-Ortega and Jan, 1991). Thus, the number of cells in the embryonic nervous system could be regulated through naturally variable activity of genes specifying stem cell fates.

Generation of ganglion cells from stem cells

How many mature cells are derived from a single precursor? The number may again be regulated by either intrinsic or extrinsic properties. In *Drosophila*, sensory organ precursors always give rise to exactly 4 cells, suggesting an innate capacity. In a group of identified cells in the CNS, a single glioblast undergoes a precisely stereotyped pattern of cell divisions, possibly reflecting an intrinsic restriction (Jacobs, et al., 1989a). The *staggerer* mutation in mice results in reduced numbers of Purkinje neurons; those remaining show aberrant morphologic traits. Analysis of *staggerer* chimeras suggests that precursor cells behave according to an intrinsic program to produce Purkinje neurons (Herrup and Mullen, 1979; Herrup and Mullen, 1981). Additional chimeric studies have used the *lurcher* mutation, which acts cell autonomously to remove all Purkinje cells. Chimeric *lurcher* mice were used to show that a single wild-type precursor cell produces about 9000 Purkinje neurons in the

C57BL/6 mouse strain and about 8000 in an AKR/J genetic background (Herrup, 1986; Herrup and Sunter, 1986). One interpretation of these results is that precursor cells are intrinsically committed to produce a certain number of progeny, but that the specific number is subject to genetic variability.

Non-autonomous determination of precursor cell capacity has been demonstrated by the experimental use of soluble agents. Retinal epithelium responds to application of thyroxine hormone with increased mitotic activity, indicating a role for endocrine function in normal neuronal development (Beach and Jacobson, 1979; Cline and Constantine-Paton, 1986). Interestingly, thyroxine application to cerebellar cortex leads to premature maturation, with the effect of reducing the total number of granule cells produced (Nicholson and Altman, 1972). This observation presages the phenotype seen in *anachronism* mutants (above), in which a premature switch from stem cell proliferation to ganglion cell production results in small, poorly organized optic lobes. The defect apparently resides not in the affected neuroblasts themselves but in the surrounding glial cells (Ebens et al., 1993).

Another non-autonomous mechanism regulating ganglion cell production may be mediated by afferent neurons. Several studies have shown correlations between axon ingrowth and cell division. Within the vertebrate central nervous system, surgical removal of a frog's eye reduces mitotic activity in the ventricular zone of the tectum (Kollros, 1982). Depletion of Purkinje neurons, as in *staggerer* mutants, affects the production of cerebellar granule cells with which they normally interact (Herrup and Mullen, 1979). Peripheral neurons also show inductive effects. Growth of retinal fibers into the brains of insects and crustaceans has been correlated with the pattern of optic lobe development (Nordlander and Edwards, 1969; Macagno, 1979; Anderson et al., 1980; Hofbauer and Campos-Ortega, 1990). This correlation is due to the mitogenic effect of retinal axons on neuronal precursors in the target field (Selleck and Steller, 1991). (Recall that although the vertebrate retina is part of the brain, the insect retina is in the PNS.)

Cell death in neural development

Much recent attention has been given to the role of regulated cell death in organismal development (for recent reviews see Oppenheim, 1991; Raff, 1992; Raff et al., 1993). Cell death is an essential feature of insect and

amphibian metamorphosis (Truman, 1984; Kimura and Truman, 1990). It is also essential for some aspects of morphogenesis, such as the elimination of interdigital webbing. Cell death is prominent in the vertebrate nervous system, where as many as half of all neurons generated may be eliminated. Matching the numbers of pre- and postsynaptic neurons by a cell death mechanism permits developmental plasticity, yet it is not obvious why such a huge excess of neurons are produced. The generation of "extra" neurons may occur as a side effect of some other adaptive process, or it may be adaptive in and of itself. Perhaps extra neurons could be utilized in new specialized functions during the course of evolution (Williams and Herrup, 1988). Regardless, it is clear that cell death plays an important role in overall control of neuron numbers.

Cells in the nematode principally acquire their fates according to successive lineage restrictions (Sulston and Horvitz, 1977). This method of fate determination is also true for "death" fates: the pattern of cell death is invariant in wild-type *C. elegans*. Analysis of mutations affecting cell death has indicated that the doomed cells initiate their own death program, and that cell death is under strict genomic control (Ellis and Horvitz, 1986; Ellis et al., 1991; Hengartner et al., 1992).

In contrast, initiation of cell death in *Drosophila* is often under epigenetic control. Elimination of cells may be triggered by hormones (Truman, 1984; Kimura and Truman, 1990) or by cell contact (Wolff and Ready, 1991). A survey of cell deaths in the embryonic nervous system revealed asymmetries in the number and position of dying cells, suggesting some plasticity in the initiation or acquisition of the death phenotype (Abrams et al., 1993). Cell death in the developing visual system illustrates the use of this mechanism in matching proportions of different kinds of neurons. Mutations that reduce the number of photoreceptors innervating the optic lobes also increase the amount of cell death in the optic lobes, affecting various precursor cell types as well as some differentiated neurons (Fischbach and Technau, 1984; Steller et al., 1987).

As discussed in the previous section, a variety of soluble factors are able to promote neuronal survival *in vitro*. Molecules of this type are central to the neurotrophic theory, which states that neurons generated in excess must compete for limited quantities of trophic factors produced by their targets. This theory fits well with observations of NGF-dependent neurons of the sensory and sympathetic ganglia (Levi-Montalcini, 1987; Barde, 1989; Oppenheim, 1991). A variation on the neurotrophic theory has also been invoked to explain massive

death of glial cells during normal development of the optic nerve. These glia can be rescued by application of PDGF and other glial-derived factors that are normally present in limited amounts (Barres et al., 1992, 1993). In contrast, studies of motoneuron death during development have suggested that the limiting reagent for neuronal survival in this system is not a trophic factor, but rather access to synaptic sites, even prior to the onset of synaptic activity (Oppenheim, 1989). Perhaps these sites are the source of spatially restricted muscle-derived or glial-derived trophic molecules.

In addition to the importance of target-derived molecules, other mechanisms may affect cell survival. For example, many cells depend on afferent fibers (Oppenheim, 1991; Raff et al., 1993). This dependence could reflect the requirement for a soluble signalling molecule as in the neurotrophic mechanism, or it may imply a direct requirement for electrical stimulation. In fact, for several experimental cases, death of target cells is increased by blocking electrical activity of afferent neurons (see Maderdrut et al., 1988). Still, this does not distinguish between the requirement for electrical vs. chemical input: the hypothetical factor may be released during synaptic activation. Alternatively, electrical activity itself may promote target survival: denervated muscle can be rescued from cell death by direct electrical stimulation of the muscle (Bloom et al., 1985).

The cumulative effects of afferent-dependent and target-dependent survival result in a finely tuned adult nervous system. Whichever cell type is limiting in a particular neuronal circuit will ultimately determine the number and proportion of other cells of various types in the circuit. In this way, precise wiring is attained, and the animal is spared the expense of metabolically supporting cells that are not functionally connected. Unfortunately, these dependencies also make the animal extremely vulnerable to small developmental defects or injuries within the nervous system. Failure of a single cell type to produce its trophic signal, or lesion of a small part of the brain, could result in the elimination of all the neurons in one or several overlapping circuits. To the extend that these circuits interact with others, the initial deficit will be amplified, carrying over to other aspects of the animal's behavior. That such amplification is characteristic of disorders such as Parkinson's and Alzheimer's disease reinforces the importance of studying molecular aspects of trophic interactions in the adult nervous system.

The adult visual system of *Drosophila* provides an attractive model for studying the molecular basis of mechanisms controlling cell numbers. It appears that *Drosophila* utilizes many of the same cellular strategies for regulation as do vertebrates, and perhaps many of the same molecules as well. Experiments in this thesis have employed genetic methods to examine the role of afferent neurons in regulating constructive and destructive events in the optic lobe. By way of introduction, the next section briefly discusses genetic analysis of, and mutations affecting, visual system development.

Mutations affecting visual system development

How many genes are required for visual system development? A survey of enhancer trap lines suggested that half of all *Drosophila* genes might be expressed in the developing embryonic nervous system (Bier et al., 1989). This estimate is somewhat questionable due to the possible differences between enhancer detection and endogenous transcript expression, a problem which is widely discussed but poorly documented. Nevertheless, it provides a starting point for assessing the level of genomic participation in neural development. Assuming that a large fraction of genes expressed in one neural tissue will also be expressed in another, it is reasonable to assume that many more genes affect visual system development than have been identified to date.

Another estimate is provided by mosaic analysis of essential genes. Starting with 68 lethal strains, Thaker and Kankel (1992) generated mutant clones in adult compound eyes. In 47 cases structural phenotypes in the retina, lamina or both could be confidently attributed to the effects of the clone. Thus *two-thirds* of lethally mutable loci in the survey were required *in retinal cells* for appropriate visual system structure! Obviously, this estimate does not refer to all genes, nor to all cell types in the visual system. Furthermore, the loci sampled may not have been representative. Still, it underscores the point that a huge number of genes may participate in the development of the visual system.

Recent screens for mutations affecting the visual system have focused on third instar larval phenotypes of mutations that confer late larval or pupal stage lethality (Datta and Kankel, 1992; Shea et al., 1994). However, most authors have only examined viable alleles and adult defects. To recover mutations with visual system phenotypes, various investigators have screened for structural changes which can be scored in the external compound eye, or for abnormal

behavior in response to visual stimuli, or for structural defects revealed in sections of adult heads. Mutations found in such screens were either in non-lethally mutable loci (such as the rhodopsin genes) or, in many cases, were not null alleles. Not surprisingly, many genes that are important for visual system development and function are also required in other tissues during embryonic and postembryonic development. For these reasons, the number of visual system mutants on record is significantly smaller than the above estimates would predict. Future investigators will undoubtedly rely on mosaic screens of lethal mutations to discover new genes acting in the visual system (Thaker and Kankel, 1992; Xu and Rubin, 1993). For the present, a brief summary of viable visual system mutations pertinent to this thesis is given in Table 1.1.

Aims of this thesis

This thesis presents progress in understanding cellular and molecular events and interactions in the visual system. The general goal of experiments herein was to address cellular and molecular mechanisms underlying retinal control of lamina development. Toward this end, I participated in a lab-wide screen for genes expressed in the developing visual system, using the enhancer trap method (Bellen et al., 1989; Bier et al., 1989; Wilson et al., 1989). This method permits screening of genetic loci by their pattern of expression rather than by examining mutant phenotypes. While not without fault, the method has the important advantage of identifying interesting visual system genes that may be essential for pre-adult survival, and marking them with a molecular tag. In addition to the enhancer trap screen, I engaged in a collaborative study of lamina glial cell generation.

Chapter 2 presents the findings of the glial cell study. We examined the relationship between retinal axon ingrowth and lamina glial cell birth and differentiation, using cell-type specific markers and metabolic labeling. These experiments led to a model for photoreceptor-dependent regulation of glial cell differentiation and survival. This chapter also includes the first attempts to distinguish lineages within the lamina, both between neuronal and non-neuronal cell types, and among different glial subtypes. The results of this analysis indicate a role for lineage restriction in regulating glial cell identity.

In Chapter 3, I describe the initial characterization of a genetic locus that is expressed in the visual system. The locus was identified in the enhancer trap

screen above. Molecular and cell biological analysis of the gene product suggests that it is a secreted molecule. Mosaic analysis indicates that it acts via a non-cell-autonomous mechanism to promote survival of neuronal targets of photoreceptor cells. This demonstrates a previously unidentified role for retinal neurons in trophic interactions in the visual system, and opens a way to study afferent-dependent trophic signalling in *Drosophila*.

Chapter 4 contains a brief recapitulation of perspectives and conclusions of the thesis, and proposes some directions for continued study. Finally, the Appendix introduces an additional genetic locus recovered in the enhancer trap screen, and describes initial efforts in its characterization.

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Figure 1.1. Structure of the adult visual system of Drosophila. Cell body layers (cortex) of the retinal, lamina, and medulla are depicted. Between the lamina cortex and the medulla cortex resides the lamina neuropil (LNP), where photoreceptors make synapses onto their target neurons. Alternating cortical and neuropil layers continue into the inner optic ganglia (not shown). Adapted from Kunes and Steller, 1993.

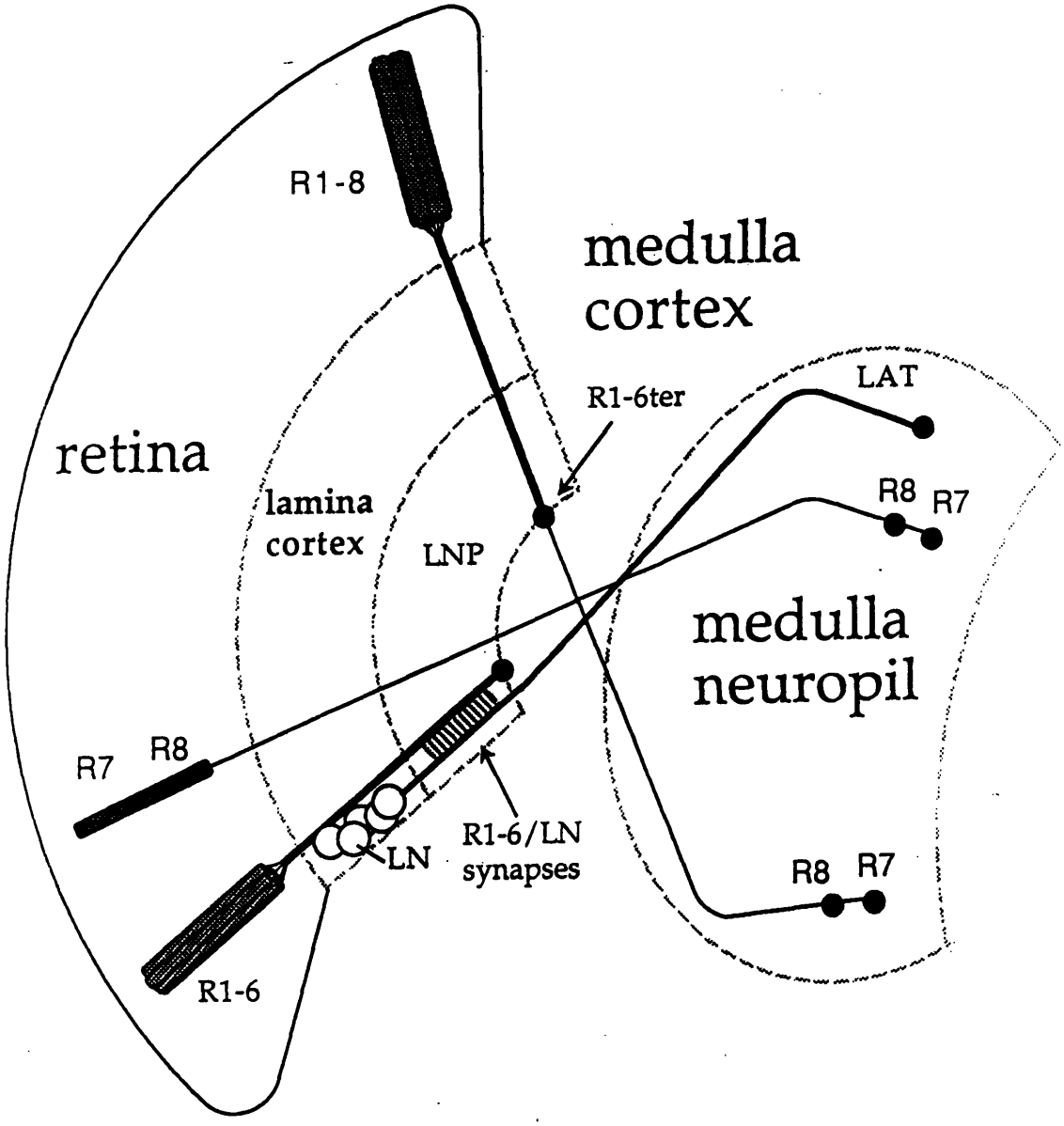

Figure 1.2. A schematic ommatidium. The lens system consists of a fluid-filled pseudocone (C) bordered laterally by the primary pigment cells (PP), basally by the cone cells (CC), and apically by the corneal lens (CL). This unit collects and focuses light onto the underlying rhabdomere (RH) which is a dense microvillus structure carrying the photosensitive pigments. The rhabdomeres of R1-R6 extend the depth of the ommatidium and are arranged in an asymmetrical trapezoid pattern. R7 provides a central rhabdomere to this arrangement in the apical part o fht eommatidium and R8 provides this function basally. A sheath of secondary and tertiary pigment cells (SP, TP) optically insulates each unit. The ommatidium has a hexagonal profile in cross section and at alternate vertices a mechanosensory bristle (B) projects to the exterior. Taken from Tomlinson, 1988.

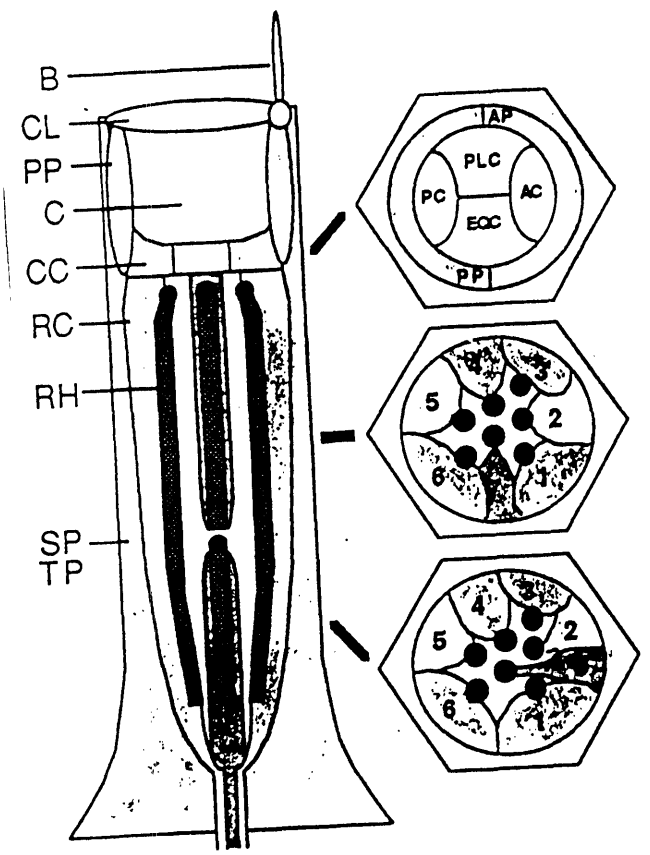

Figure 1.3. A schematic cartridge. A cluster of 5 lamina neurons is insulated by satellite glia in the cell body layer, epithelial glia in the neuropil layer, and marginal glia at the basal interface with the medulla. Photoreceptor neurons R1-R6 project the length of the neuropil region, where axons are thick with synapses and specialized junctions with glia, and terminate above the marginal cells. R7 and R8 (not shown) extend through the neuropil layer outside of the cartridge, between the glia. Lamina monopolar neurons extend into the external medulla neuropil (not shown).

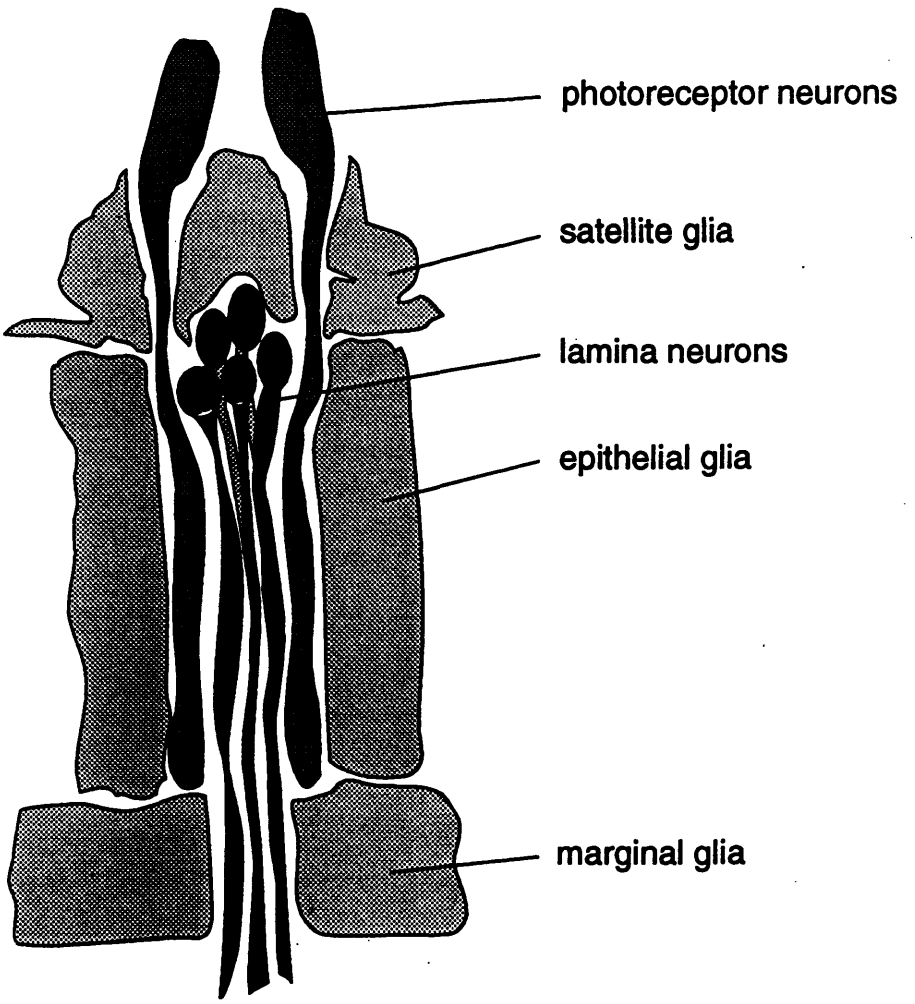

Figure 1.4. Schematic longitudinal section of the developing eye disc. Anterior is to the left. Cells extend the full apical to basal range of the disc except during mitosis, when they round up and move apically. Long section provides a developmental timecourse. As cells are successively recruited into ommmatidia, different individual cell types express different antigens. Consequently clusters in different positions along the anterior-posterior axis show age-dependent expression. For example, clusters at stage II can be stained with boss and rough antibodies. At stage III clusters can also be stained with andtibody against seven-up. Roman numerals indicate stages, arabic numerals indicate photoreceptor cell types. C, cone cell. mf, morphogenetic furrow. Diagram taken from Tomlinson, 1988.

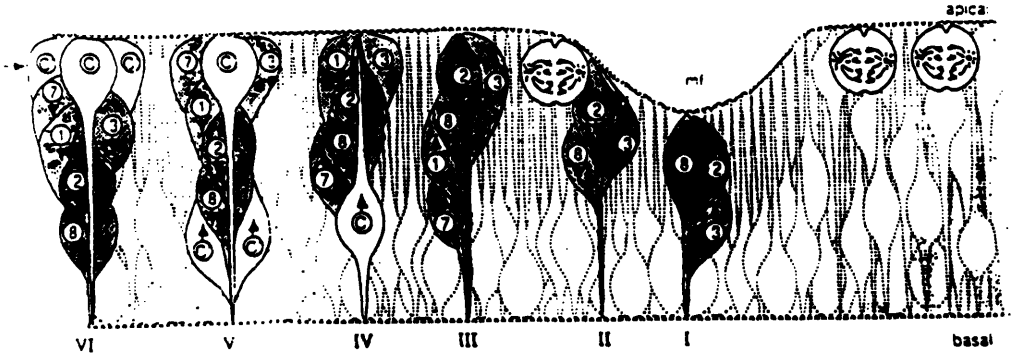

Figure 1.5. The inner and outer proliferation centers of the developing optic lobe. These structures resemble a rod surrounded by a ribbon, which expand and pull away from each other during the larval growth period. The outer proliferation center sheds cells from its lateral (toward the lamina) and medial (toward the medulla) faces. The inner proliferation center generates the lobula and lobula plate. Differentiation of optic lobe cortex and neuropil in the intervening space begins before proliferation has been completed at the margins.

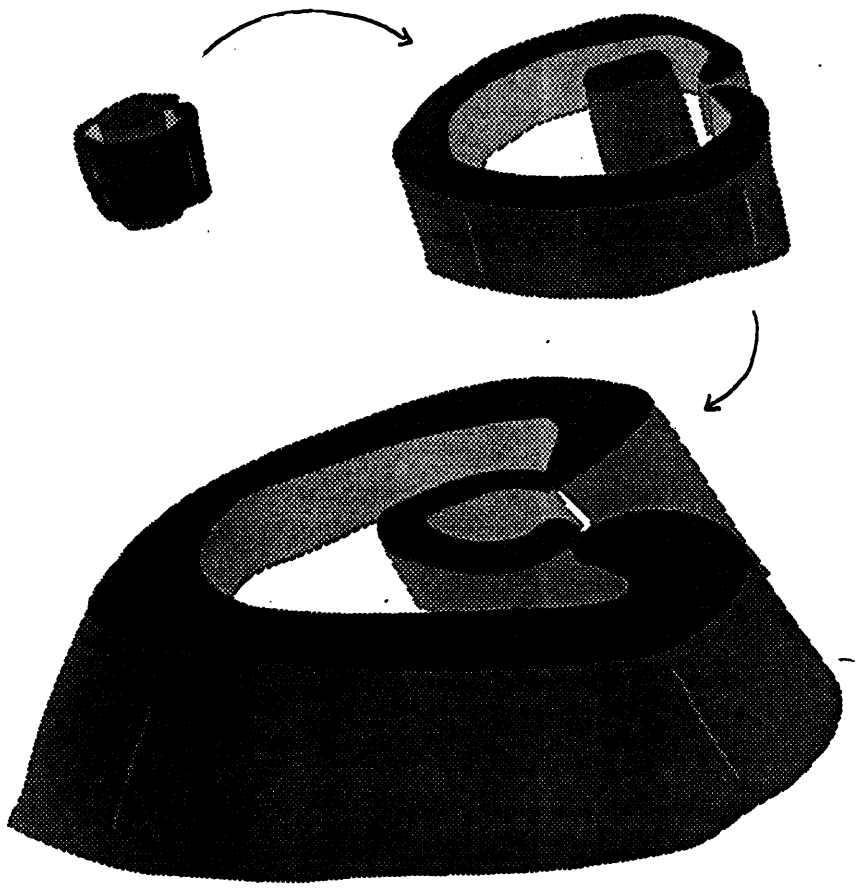

Table 1.2 A partial list of mutations affecting structure of the visual system. Much of this is taken from Lindsley and Zimm, *The Genome of Drosophila melanogaster.* Additional information has been gleaned from lecture notes and meeting abstracts, especially the 1993 *Neurobiology of Drosophila* meeting at Cold Spring Harbor.

Mutations affecting eye size or retinal projections

ato *atonal.* Rare adult escapers of embryonic lethality are eyeless and have small optic lobes. bHLH- type transcriptional regulator.

chp *chaoptic.* Rhabdomeres highly deranged to absent in different alleles. Adjacent retinular cells not correctly apposed. Glycoprotein expressed on surfaces of photoreceptors in retina and along axons.

disco *disconnected.* Retinal fibers of most eyes do not project to the optic lobes, but in other respects ommatidia develop normally. Unconnected retinas degenerate over time. Probable transcriptional regulator with two Zn fingers.

Elp *Ellipse.* Eyes of heterozygotes rough and oval. Homozygotes have smaller eyes with many fewer ommatidia than normal, but those ommatidia which are present have the normal complement of cells. Defect presumed due to aberrant specification of clusters in third instar eye disc. Hypermorphic allele of the Drosophila EGF receptor.

eya *eyes absent.* Weak alleles result in massive death of cells in the third instar eye disc, anterior to the morphogenetic furrow. Strong alleles affect a greater variety of cell types and strongest alleles are lethal. Homeobox-containing nuclear protein.

ey^{D} *eyeless Dominant.* Eyes of heterozygotes small and ommatidia irregular. Often duplicated antennae or ocelli. Cell death in second instar discs. Homozygous lethal. May be a homeobox containing gene.

gil *giant lens.* Adult ommatidia show incorrect cellular composition. Pattern of photoreceptor projections in the optic lobe aberrant in third instar, possibly due to abnormal glial cell development. Novel secreted protein expressed in both eye disc and optic lobe.

gl *glass.* Retinal neurons lack rhabdomeres and flies are not photosensitive. Small eyes with reduced pigmentation and glassy surface. Abnormal retinal projections; in strong alleles the lamina does not form, medulla is small and disorganized. Transcription factor with 5 Zn fingers.

so *sine oculis.* Compound eye reduced or absent due to death of cells in developing eye disc. Lamina and outer medulla correspondingly reduced. Stronger alleles show additional autonomous defects in the brain.

Mutations affecting optic lobe development

ana *anachronism.* Severe disorganization of the optic lobes, apparently resulting from incorrect timing of optic lobe neurogenesis and decreased cell number. Glycoprotein secreted by glial cells surrounding the optic lobe.

disco *disconnected.* Optic lobes disorganized more than can be accounted for by unconnected eyes, possibly reflecting requirement for disco in optic lobe pioneers or in lobula and medulla neurons. Occasionally muscle cells found under the retina.

drd *drop-dead.* Superficial normal structure at eclosion, but glial cells show aberrant morphology: stunted processes fail to wrap around neurons. Degeneration and early adult lethality. Novel protein, probably secreted from cells surrounding neuropil regions in the optic lobes and central brain.

mnb *minibrain.* Volume of optic lobe reduced in adult, though organization apparently normal. Neuronal cell death in pupal optic lobes. Protein kinase, serine/threonine type, expressed in optic lobe neuroblasts.

omb *optomotor-blind* gene complex. Reduced optomotor response to large field rotary visual stimuli due to missing neurons in the lobula plate. Inner optic chiasm deformed. Abnormal ERG may be due to defective tangential elements in the lamina. Novel DNA-binding protein.

opt *opthalmosa.* Optic lobe anlagen fails to invaginate during embryonic development. Extra cell death in third instar eye disc. Homeobox-containing gene.

repo *reversed polarity.* Abnormal ERG suggestive of poor electrical isolation of the retina. Abnormal differentiation of lamina glia. Homeobox containing gene expressed exclusively in glia.

sol *small optic lobes.* Reduced volume of medulla, lobula, lobula plate due to death of specific cell types during pupation. Abnormal phototaxis and geotaxis. Zn finger containing protein expressed in glia.

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

Preface

Experiments in this chapter were accomplished through a collaborative effort with Sharon Perez, who is also a graduate student in the laboratory of Hermann Steller. In particular, the birthdating analysis involved significant contributions on both our parts. I developed the method for topical application of BrdU for *in vivo* labelling, and performed the mosaic analysis using the VP19 reporter.

This chapter has appeared previously as

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.

Summary

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

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.

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. Figure 2.1A-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 Figure 2.1B (and magnified in Figure 2.1C), sections are also stained with anti-ELAV antibody (black nuclei); the two antigens do not overlap. Together, these markers account for essentially all of the nuclei that are detected by bis-benzimide staining and that have been described in the adult lamina (Strausfeld, 1976). The reporter is also expressed in an additional layer between the retina and the lamina, named the subretinal cells. Finally, expression is detected in non-neuronal cells, presumably medullary glial cells, which surround the medulla neuropil.

3-109 marks lamina glial cells in third instar larvae.

Given the specificity of the adult expression pattern, we asked whether 3-109 also specifically marks glia in the developing third instar lamina, at the time when cellular differentiation begins. We found that 3-109 is expressed in a restricted set of cells in the larval brain (Figure 2.1D-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. Figure 2.1D 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 Figure 2.1E and F, two focal planes of the same 3-109 third instar larval brain are shown labelled with anti-β-galactosidase and anti-ELAV antibodies. (Due to the curvature of the tissue, a single focal plane is insufficient to view all the pertinent cell types.) Note that the satellite glial nuclei are positioned between the lateral (L1-4) and medial (L5) lamina neurons. These panels also indicate β-galactosidase expression in the medulla glia, positioned between the marginal glial cells of the lamina and the underlying medulla neuropil. These medullary cells are distinguished from the L-glia not only by their position but also by the characteristic disc shape of their nuclei. Finally, expression is detected in the subretinal cells. At this time, most of the subretinal layer still resides in the eye disc, from which these cells are derived (Cagan and Ready, 1989); only a few cells have crossed to the lateral margin of the brain.

Expression of glial cell markers depends on retinal innervation.

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

The expression pattern of 3-109 in a wild-type animal is shown in Figure 2.2A. 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 (Figure 2.2B). 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 Figure 2.1). 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: Figure 2.3B shows a section from one individual in which all the L-glia have retained reporter expression, but L-neurons are unstained. Figure 2.3C 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). Figure 2.4A-C and 2.4D-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 (Figure 2.4G), 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 (Figure 2.4H). 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 2.2. 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 Figure 2.4G, 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.

Figure 2.5A-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 Figure 2.4H). 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 Figure 2.5D, 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 Figures 2.4H and 2.5C). 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 Figure 2.1D). 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 Figure 2.4A-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 R-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 Figures 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.

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/+; Δ2-3, 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^{o}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^{o}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^{o}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 μg/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/*CyO*, 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.

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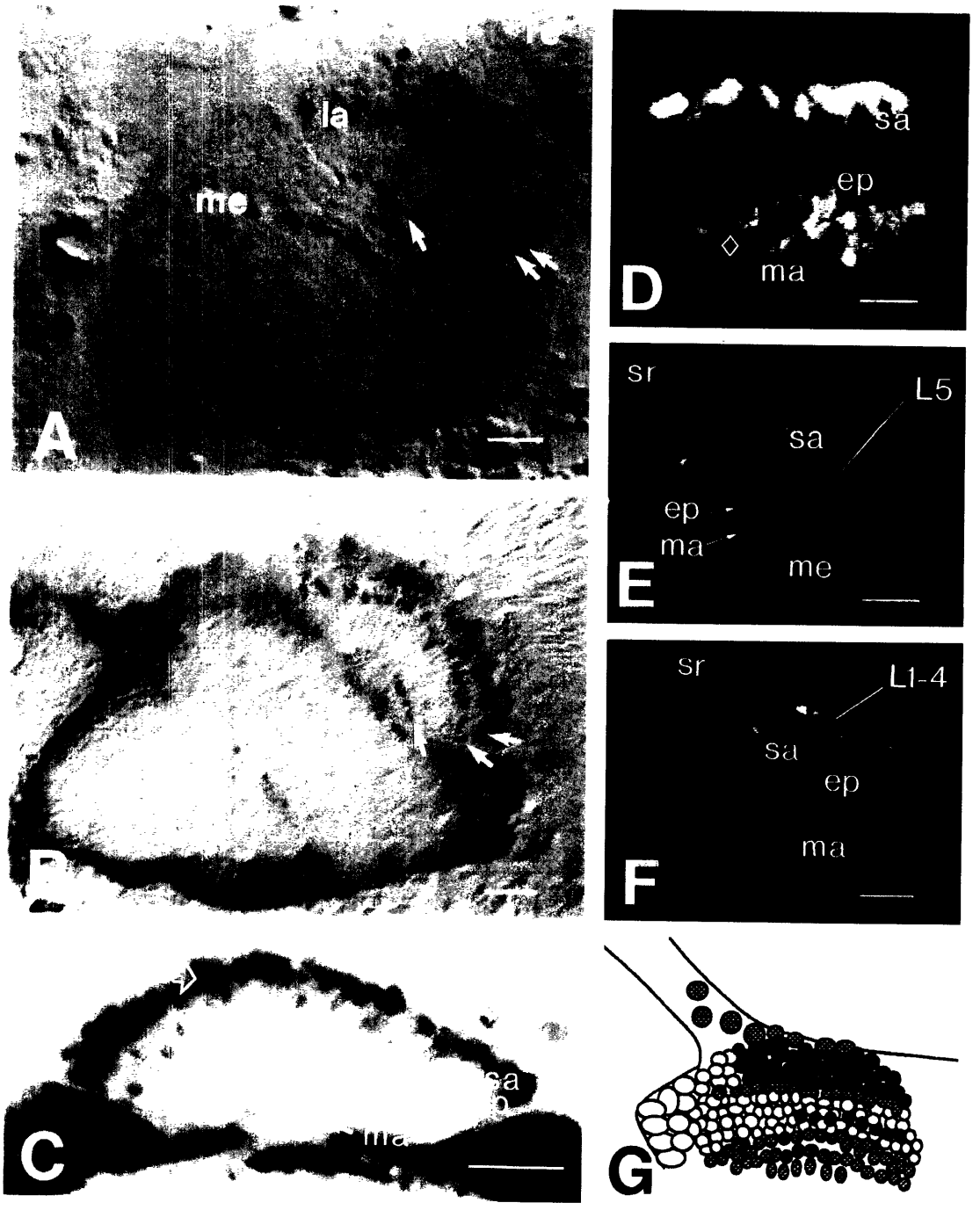
Figure 2.1. Expression of 3-109 in wild-type animals. (A) Adult head section showing relative positions of the retina, lamina and medulla. Glial nuclei (brown) are detected with anti-β-galactosidase antibody, L-glia are indicated with arrows. (B, C) Adult head sections stained with anti-β-galactosidase antibody (brown nuclei) to detect glia and anti-ELAV antibody (black nuclei) to detect neurons. L-neurons are indicated with an open arrow, L-glia with closed arrows. (D-F) Confocal micrographs of whole-mount, third instar larval brains stained with anti-β-galactosidase antibody (green nuclei) to detect glia, and with either anti-HRP antibody (in red, panel D) to visualize photoreceptor axons or with anti-ELAV antibody (red nuclei, panels E, F) to detect neurons. The layer of photoreceptor 1-6 termini is indicated by a diamond in panel D. (G) A schematic representation of a third instar lamina, with glial nuclei in gray and neuronal nuclei in black. Anterior is up in A and B. Panel D shows a frontal section with lateral up. Anterior is to the left and lateral is to the top of the page in E-G. ep, epithelial glia; la, lamina; L1-4, L5, lamina neurons; ma, marginal glia; me, medulla glia; re, retina; sa, satellite glia; sr, sub-retinal cells. Scale bars, 20 μm.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G

Figure 2.2. 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.

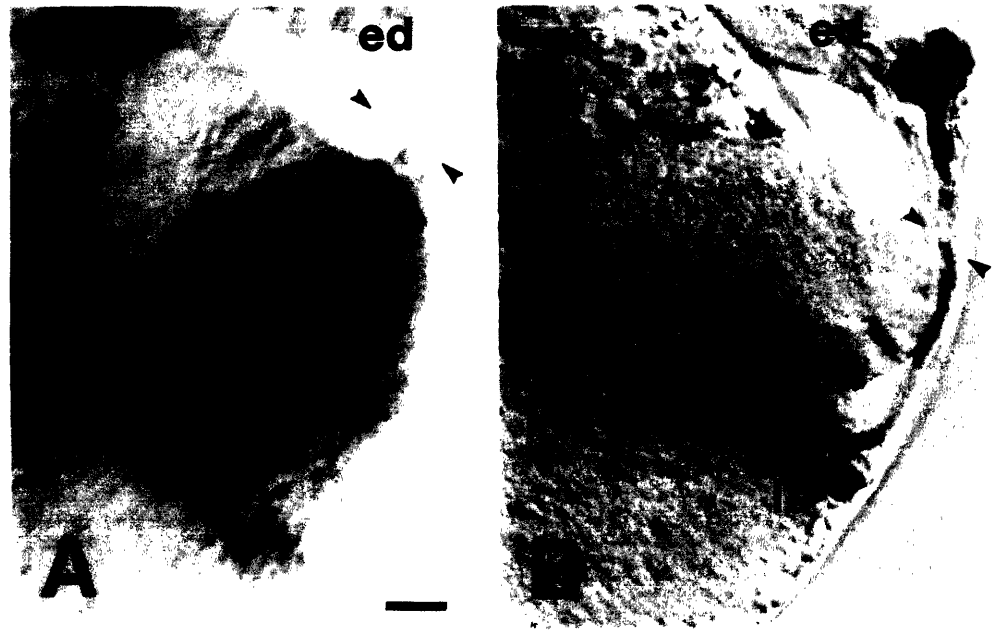

ed
A

Figure 2.3. 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 that has lost reporter expression in most L-neurons while retaining expression in L-glia is represented in panel B. An animal that has lost reporter expression in L-glia while retaining expression in most L-neurons is represented in panel C. Arrows indicate the border between the lamina (left) and the retina (right). Anterior is to the top of the page. Scale bars, 20 μm.

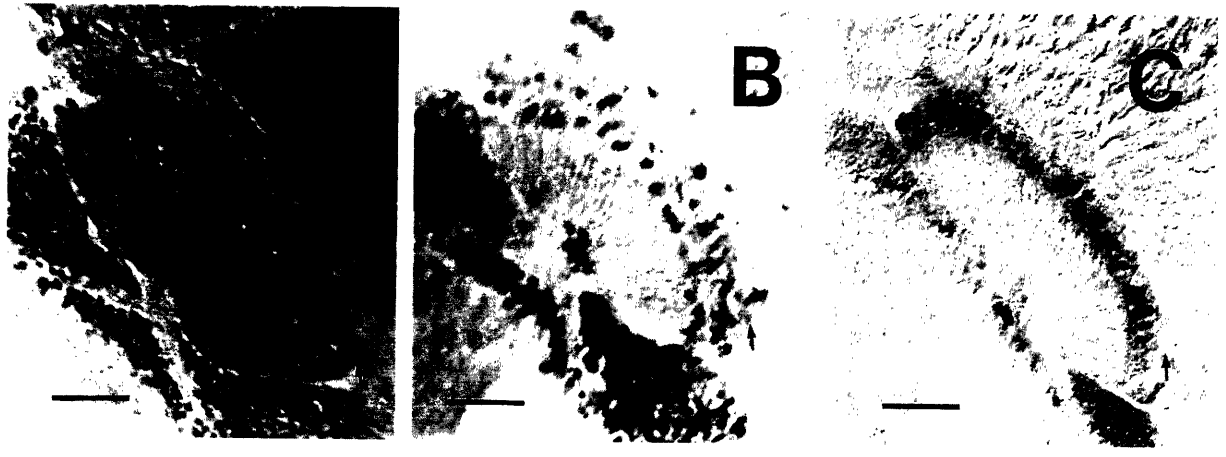

B
C

Table 2.I(A). *Somatic mosaic analysis of lamina glia and neurons, using a ubiquitously expressed VP19 reporter as the target for excision.*

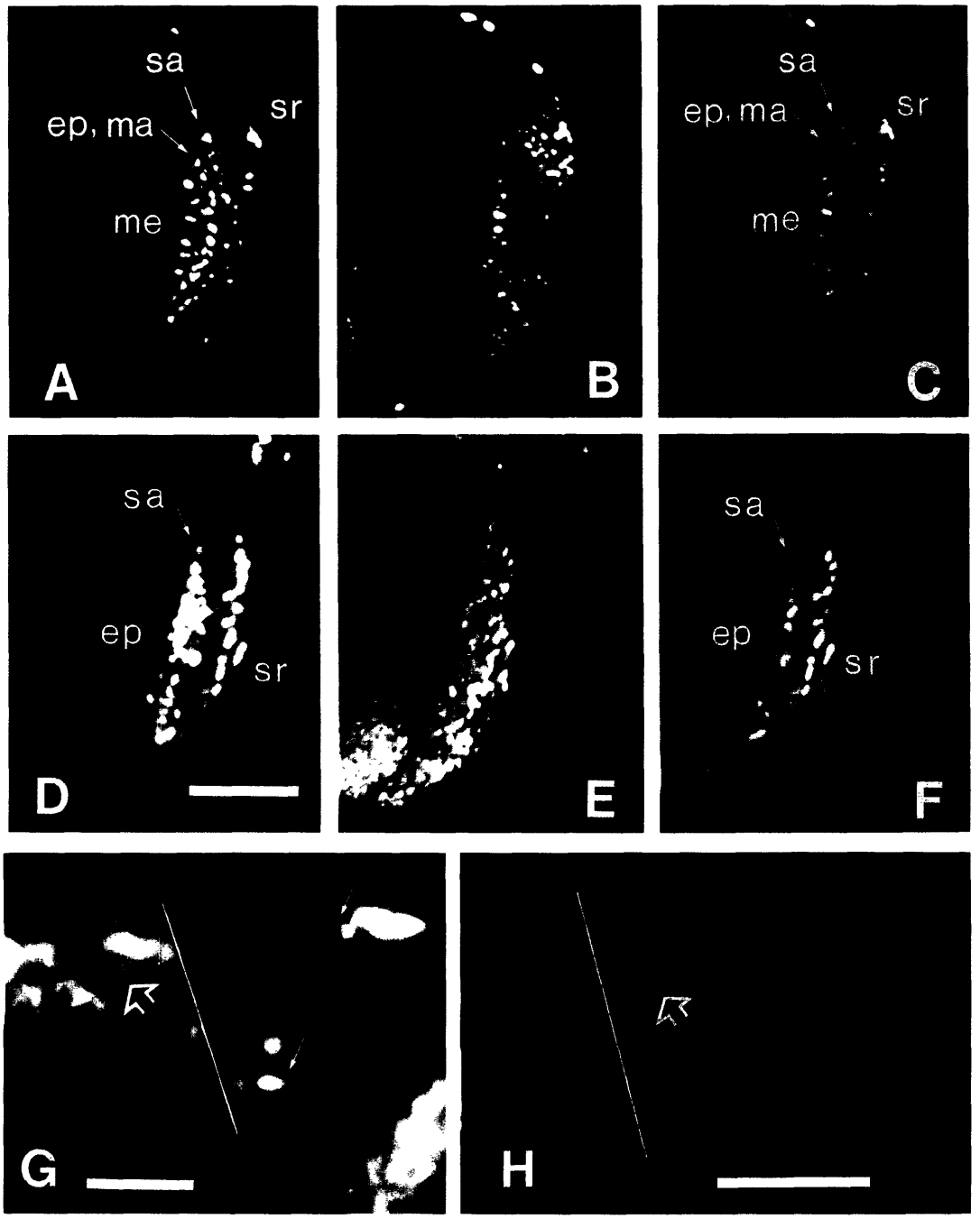
Optic lobes examined			65
Total number of patches affecting optic lobes			56
- optic lobes containing one lacZ-negative patch		30	
- optic lobes containing two lacZ-negative patches			13
Patches confined to the lamina		37	
- confined to lamina neurons	8		
- confined to lamina glia	29		
- crossing L-neuron 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.

Figure 2.4. 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 Figure 2.1. Magnification is the same for A-F. Scale bars, 50 μm.


sa
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F
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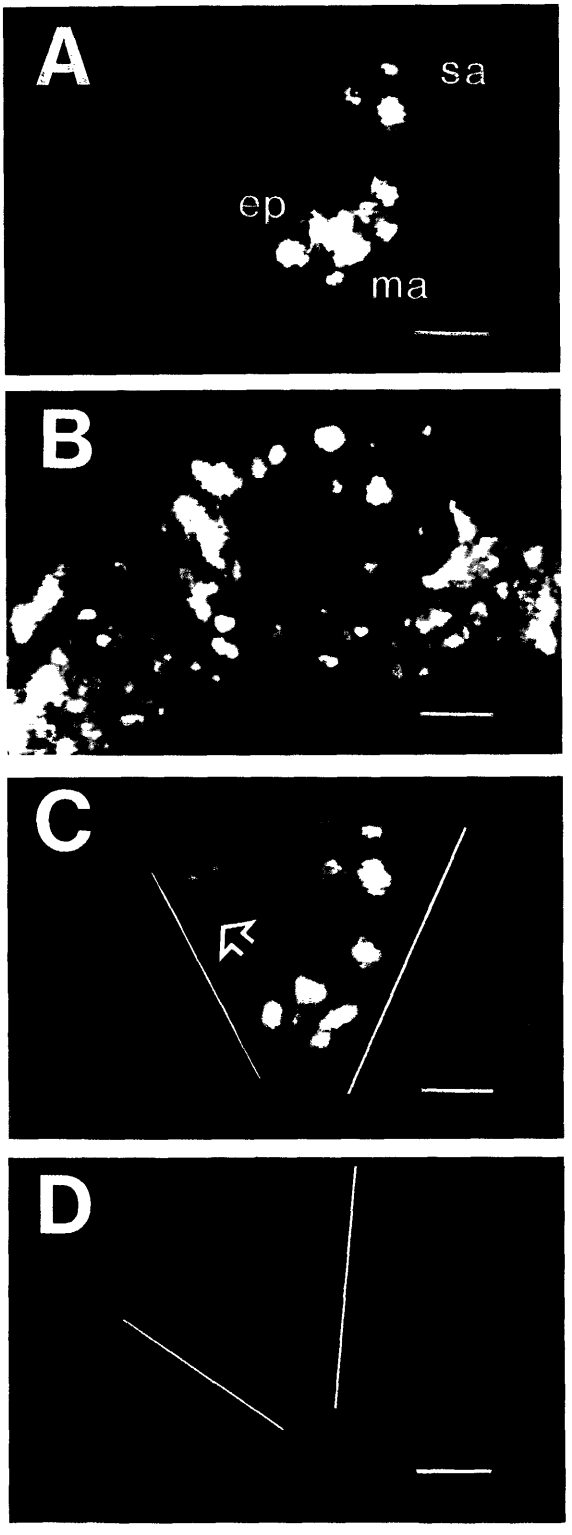
G
H

Table 2.2. *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

Figure 2.5. Long BrdU pulses in late third instar. Whole-mount, late 3IL brains were stained with anti-β-galactosidase antibody (panel A, green/yellow nuclei in panels C, D) and anti-BrdU antibody (panel B, red/yellow nuclei in panels C, D) and viewed by confocal microscopy. Panels A-C represent a single wild-type animal. (A) Glial cells. (B) Cells which took up BrdU during the 6-hour pulse, including extensive incorporation in the proliferation zones (right and left) and restricted incorporation into the developing lamina (center). (C) Yellow indicates co-localization; many of the satellite, epithelial and marginal glial cells are BrdU-positive. Products of LPC divisions have chased into the developing lamina (open arrow). (D) A long BrdU pulse in a *sine oculis;* 3-109 animal lacking retinal innervation. No β-galactosidase was detected. Borders of the developing lamina are given by white lines in panels C, D. Anterior is to the left and lateral to the top of each panel. Abbreviations as in Figure 2.1. Scale bars, 20 μm.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Chapter III: The *incomunicada* locus is required for the survival of cells in the *Drosophila* visual system

Summary

Nervous system assembly requires coordination between neurons and their targets to achieve correct numbers and proportions of different cell types. In *Drosophila*, retinal neurons induce the birth of their target interneurons during optic lobe development. Here we present evidence of an additional role for retinal neurons in long-term trophic interactions. We describe a genetic locus, *incomunicada*, that is required in retinal neurons for the survival of their targets in the lamina. Phenotypes in these and other tissues are consistent with non-cell-autonomous activity of the gene product. Sequence analysis of the *incomunicada* gene predicts a novel secreted protein; antibody staining indicates vesicular axonal transport. The combination of phenotypic and expression analysis suggests that *incomunicada* may directly mediate interactions between cells of the visual system to promote their survival.

Introduction

A major task for the developing nervous system is that of matching numbers of presynaptic and postsynaptic elements. This problem arises because neurons and their targets often arise from separate precursors, and may be born at large distances from one another. One mechanism for matching the number of neurons to the size of their target field is described in the neurotrophic theory, based largely on the study of nerve growth factor (NGF) and its relatives (reviewed in Levi-Montalcini, 1987; Raff et al., 1993). This theory holds that neurons require, and compete for, survival factors that are produced by their target cells. Input neurons are generated in excess; those failing to find synaptic partners presumably do not receive the necessary factor and are eliminated. Target-dependent survival, or retrograde dependence, has been best studied in vertebrates, but insect species also employ this strategy (Bacon and Blagburn, 1992). Afferent-dependent, or anterograde, survival interactions have also been described. The best-known example comes from

the vertebrate neuromuscular junction, in which survival of target muscle is mediated both by electrical stimulation and by neuro-transmitters released from motoneurons (reviewed in Purves and Lichtman, 1985). Although many cases of transneuronal afferent-dependent interactions have been reported, in these cases the molecular nature of the "trophic interaction" is unknown (reviewed in Oppenheim, 1991).

In the *Drosophila* visual system, size of the compound eye is variable, yet the target field in the optic lobe is always appropriately matched (Power, 1943). This is partly due to progressive events: e.g., growth of retinal axons into the developing optic lobe induces production of target neurons and differentiation of nearby glial cells (Selleck and Steller, 1991; Winberg et al., 1992). In addition, there is evidence for trophic activities to promote survival of visual neurons in the adult. In the *sine oculis* mutant, for example, flies may completely lack the compound eye and outer visual ganglia. Over time, specific neurons in the inner ganglia degenerate, presumably due to the lack of neuronal input (Fischbach and Technau, 1984). Another mutant, *disconnected*, has been used to demonstrate that retinal cells whose axons fail to reach the optic lobes also degenerate over time (Campos et al., 1992). These mutations begin to reveal the potential complexity of retrograde and anterograde interactions between the retina and optic lobes, and demonstrate the power of genetic analysis in this system. However, these studies have not resolved which cell types mediate survival decisions, nor identified any candidate molecules that may participate in a trophic mechanism.

We have used the enhancer trapping method (Bellen et al., 1989; Bier et al., 1989; O'Kane and Gehring, 1987; Wilson et al., 1989) to search for genes that may play a role in coordinating structural aspects of the eye and optic lobes. Here we present the characterization of a genetic locus in *Drosophila* that apparently mediates trophic interactions in the visual system. Mosaic analysis of mutations at the *incomunicada* locus has led to observation of both retrograde and anterograde effects: the gene is required in the eye to prevent degeneration of optic lobe neurons, and is likewise required in the brain for the survival of the retina. Molecular characterization of the *incomunicada* gene and gene product indicates that it is a novel extracellular protein that may act in cell-cell signalling. In the visual system, expression and phenotypes together suggest that *incomunicada* may directly mediate transneuronal trophic interactions.

Results

We screened enhancer trap lines for expression patterns that would reflect genes involved in early stages of visual system development. One line, IV27b, was selected because, during late third instar larval stage, the expression of its lacZ reporter is confined to the developing first optic ganglion, or lamina. Lamina expression of this reporter depends upon photoreceptor ingrowth for induction, consistent with previous reports genes and markers expressed during lamina development (Selleck and Steller, 1991; Winberg et al., 1992). During pupal stage, IV27b is expressed not only in the lamina, but also in retinal neurons and a subset of cells in the medulla, or second optic ganglion (Figure 3.1). LacZ expression remains high in these tissues until near the end of pupal development, when levels fall in both the retina and the optic lobe. This parallel decrease led us to speculate that the IV27b element might reflect the expression of a gene that would influence or depend upon coordinated develpmental processes between these adjacent ganglia, and we sought to characterize the corresponding locus.

Genetic analysis of IV27b.

To search for a gene near the IV27b insert, which might be required for visual system development, we mapped the P element by *in situ* hybridization to polytene chromosomes (Pardue, 1986); the element was detected in band 1E4 on the X chromosome. This genomic region has been examined previously, and many alleles of several lethal complementation groups are available (Lefevre, 1981; Lefevre and Watkins, 1986; Perrimon et al., 1989). We generated mutations in the genomic locus surrounding the insertion site by remobilizing the IV27b element to create imprecise excisions (Daniels et al., 1985; Tsubota and Schedl, 1986). Excisions were detected by the loss of the wild-type *rosy* gene carried by the P element. None of the viable strains thus generated showed any obvious phenotypes, even though many involved small deletions around the point of insertion (see below). In addition, a number of recessive lethal lines were generated. These were initially screened by crossing to a genomic duplication that covers the IV27b insertion site. Five of the lethal mutations were also covered by the duplication, making them

candidates for deletions in the presumptive IV27b locus (Figure 3.2). These 5 candidate lines were tested for their ability to complement each other, as well as representatives of previously described lethal complementation groups in the interval. We found that 3 lines corresponded to the *l(1)1Ef* locus, and one line corresponded to *l(1)1Ec*. [ed. note: The notation *l(1)1Ef* indicates lethal complementation group on chromosome 1 mapped to interval 1E and arbitrarily assigned to group f. The final letter is not intended to convey relative position within the 1E region.] The fifth line failed to complement mutations in both of these groups, suggesting that it may be a deletion uncovering both loci. Subsequent molecular analysis has indicated that the two complementation groups correspond to mutations on opposite sides of the P element insertion site (see below and Figure 3.5).

Identifying defects in the visual system.

In order to examine whether these two lethal complementation groups play essential roles in the visual system, we performed mosaic analysis of multiple alleles from each locus. Animals with mutant tissue confined to the visual system are expected to survive to adulthood, enabling detailed structural examination of potential defects. We generated mosaics by X-ray irradiation of larvae, to create somatic clones of homozygous mutant cells. Because the IV27b reporter is expressed in retinal cells, we asked whether mutant eye patches would result in any detectable phenotypes. The closely-linked cell-autonomous *white* gene was used to mark retinal clones of IV27b excision mutations. The analysis of alleles of *l(1)1Ec* will be described elsewhere. The analysis of *l(1)1Ef*, which we have named *incomunicada*, indicated that this complementation group is important for maintaining the integrity of cells in the visual system, encouraging us to study the mutation in greater depth.

We examined flies with mutant retinal clones both early and late with respect to eclosion (adult emergence from the pupal case). Retinal neurons project into the lamina with point-to-point specificity (reviewed in Banerjee and Zipursky, 1990; Kunes and Steller, 1993). This structure makes it possible to determine what region of the lamina should be innervated by mutant photoreceptors. The specimen shown in Figure 3.3B is representative of young animals bearing retinal *l(1)1Ef* mutant clones: within the first week after eclosion, the clone and all the surrounding tissue appear structurally normal, in that the correct numbers and disposition of cells are found in the retina and

adjacent lamina. This indicates that retinal induction of lamina cell fates has occurred normally. In contrast, a similar animal that was aged for 3 weeks following eclosion shows a striking defect: although the mutant tissue (i.e. the unpigmented region of the eye) appears normal, part of the underlying lamina is severely degenerated (Figure 3.3C). Examination of over twenty mosaic animals aged in this way indicates that the lamina is affected only in precisely that region to which the mutant photoreceptor neurons project. Furthermore, the degeneration within the lamina is apparently restricted to the interneurons that are synaptic targets of retinal neurons. Glial cell nuclei can still be found in the degenerated area. We believe that the lamina neurons have degenerated due to the failure of mutant retinal neurons to provide a locally-acting trans-neuronal survival signal. This suggests that in addition to inductive signals for lamina development, photoreceptors provide trophic signals to their neuronal targets. The effects of these two signals can be genetically separated by mutations in *l(1)1Ef*. We propose the name *incomunicada*, or *inca*, to reflect the failure of cell interactions required for long-term survival in this mutant.

We also wanted to examine the role of *inca* in the optic lobes. We were especially interested in knowing whether lamina neurons, which are dramatically affected by eye clones, would show an autonomous requirement for the gene. To mark the genotype of cells in the brain, we used a LacZ reporter construct (with a nuclear localization sequence included) on the wild-type chromosome. (Unfortunately, the reporter is ubiquitously expressed for only a few days after eclosion.) Cells that lack the construct due to a somatic recombination event should also lack wild-type *inca* gene function. Antiserum against beta-galactosidase was used to identify wild-type cells; mutant cells were detected by counterstaining with a fluorescent nuclear dye. We recovered several animals with mutant patches in lamina neurons; these cells had wild-type morphology, at least at the level of light microscopy. In contrast, it appears that lamina glia require the gene for survival: the specimen in Figure 3.3E lacks many glial cells, of the epithelial glial subtype, whose cell bodies reside within the lamina neuropil. All of the cells that are present in the head are positive for LacZ and are presumably phenotypically wild-type with respect to *inca* expression. This suggests that the missing glial cells were themselves mutant. Approximately 10% of irradiated flies examined showed this defect, which was not seen in control flies. Figure 3.3F shows a similar example in a plastic preparation; here one sees that the neural organization is relatively undisturbed

despite missing cells (note that this section has not been stained for beta-galactosidase). Additional specimens have been recovered that are missing small numbers of cells representing other glial subtypes in the lamina (Figure 3.3G, H). These results indicate a cell-autonomous requirement for inca for the differentiation or survival of glial cells in the adult visual system.

Interestingly, a few of the animals that were recovered showed massive age-dependent retinal degeneration, as well as degeneration in the lamina, even though the retina was completely wild-type with respect to *inca* expression (Figure 3.3I). Although it has not been possible to assess directly the genotypes of brain cells at this late stage (because the reporter is not expressed late), we believe these animals reveal a role for *inca* in retrograde, as well as anterograde, survival interactions. Interestingly, degeneration in the retina appears to affect all classes of photoreceptor cells (Figure 3.3J). Furthermore, the glial cytoplasmic staining in many areas of the lamina suggests that degeneration was not caused by a lack of glial cells, and by inference, not by a lack of *inca* function in glial cells (see above). Together, these observations may indicate that the focus of the defect in these animals resides outside of the lamina. The low incidence of specimens showing this phenotype may correlate with the low incidence of large clones, or it may reflect the infrequency of including a particular cell type in the mutant clones. It is also possible that the phenotype described is due to an X-ray induced defect, however, retinal degeneration has not been observed in control flies.

In sum, analysis of *incomunicada* clones in the visual system has provided evidence for both retrograde and anterograde trophic activities, which can be disrupted by mutations at this locus.

Phenotypes of *inca* mutant embryos.

All of the *inca* alleles in the present study confer lethality late in embryogenesis. We examined mutant embryos for defects that would reveal the role of *inca* in normal embryonic development. Mutant embryos appear to develop quite normally for several hours after egg deposition. In particular, no obvious defects were found in peripheral or central nervous tissues. The most striking visible defect is in the structure of the tracheal network (Figure 3.4). Embryos in Figure 3.4A and 3.4B have been stained with monoclonal antibody 2A12, which stains the lumen of the trachea beginning at about 10 hours after egg laying (Fujita et al., 1982). In contrast to wild-type embryos that display an

elaborately branched network, staining in mutant embryos shows only a single dorsal trunk. Examination of older embryos indicates that the mutant trachea never fill with air (Figure 3.4C, D). In wild-type development, the trachea serve as substrates along which motor and sensory neurons grow to reach their targets (Hartenstein, 1988; Johansen et al., 1989). Motor neurons in *inca* mutants are able to reach and innervate bodywall muscles despite aberrant tracheal morphology, and mutant embryos initiate normal muscular peristalsis prior to the lethal phase. Likewise, sensory axons successfully project to the CNS. This is consistent with the report that trachea are not essential for sensory axon pathfinding (Younossi-Hartenstein and Hartenstein, 1993).

Cloning the *inca* locus.

Genomic DNA adjacent to the IV27b insertion was recovered by plasmid rescue (Steller and Pirrotta, 1986) and used to initiate a chromosomal walk. Portions of the walk were used to probe genomic Southerns of lethal and non-lethal lines derived from excising the IV27b P element, as well as 2 X-ray induced alleles of *inca*. The results are summarized in Figure 3.5. Most of the non-lethal lines appeared to be precise excisions or internal deletions of the IV27b insert. A few showed small (less than 2kb) deletions flanking the insertion site. Frozen sections from these adults were examined for structural abnormalities in the optic lobes and retina; no defects were found. The 3 *inca* alleles showed clustered rearrangements: $inca^{1}$ and $inca^{C24}$ are small overlapping deletions, and $inca^{GA91}$ is a translocation with one breakpoint in the interval. (The GA91 allele was previously recognized as an X-to-3 translocation.) A 12.5 kb genomic fragment spanning these rearrangements was reintroduced into the fly genome using P element mediated germline transformation (Rubin and Spradling, 1982). The construct, named P[*ry*, *inca*-12.5], was tested in the background of the $inca^{1}$ mutation, a presumed null allele. Adult $inca^{1}$; P[*ry*, *inca*-12.5] flies were obtained, indicating rescue of lethality. These were aged 30-35 days after eclosion and then examined in thin sections. Rescued flies show the normal visual system configuration, and no excess of degeneration compared to aged wild-type flies (Figure 3.3L). Therefore all the essential sequences of the *incomunicada* gene must be contained within this construct, and the possibility of a second confounding mutation is ruled out. Two transcribed areas have been found in the interval. The first overlaps the rearrangements and is completely contained within the

construct. This position, together with subsequent molecular analysis, makes it the best candidate for the *inca* gene. A cDNA from the second transcription unit hybridizes to genomic sequence outside of the construct; in fact, only about half of the transcribed region is contained within the rescue contruct. Moreover the transcribed interval is not affected in the *inca* rearrangements. For these reasons we assumed that this second transcription unit did *not* correspond to *inca*, and we did not characterize it further.

The 2.2 kb HindIII fragment that detected rearrangements in 3 *inca* alleles was used to screen a cDNA library made from 7-12 hour embryo RNA (Zinn et al., 1988). A single 2.1 kb cDNA was recovered; when used as a probe for Northern analysis this detects a transcript of about 6 kb, which is most abundant during late pupal development (Figure 3.7). The cDNA appears to contain the 5' third of the transcript, truncated within the major open reading frame and lacking a poly-A tail. Additional library screens were attempted in order to recover longer cDNAs, but these efforts were unsuccessful. Therefore much of the sequence data presented in Figure 3.6 is derived from genomic clones in the interval.

Structure of the *inca* gene.

The *inca* locus spans at least 8 kb of genomic sequence. Three identified exons are spliced to produce mature transcripts. The 5'-most exon is entirely untranslated. The short second exon contains a consensus translation initiation site (Cavener, 1987); the resulting reading frame remains open in the third exon for a total of 3.8 kb. A consensus polyadenylation site is located 260 bases downstream from the end of the open reading frame. Conceptual translation predicts a protein of 1267 amino acids. The N-terminus is highly hydrophobic and conforms to consensus requirements for membrane translocation and cleavage of a signal sequence (von Heijne, 1986). The algorithm of Kyle and Doolittle (1982) finds no other hydrophobic region of significant length, suggesting that *inca* is not an integral membrane protein. The protein contains 5 potential sites for N-glycosylation, and several clusters of basic residues. In some proteins, basic sequences are implicated in binding to heparin or heparan sulfate (Jackson, 1991). In other proteins they serve as targets for proteolytic processing in a regulated secretory pathway (Proudfoot and Brownlee, 1976; Hosaka et al., 1991).

The P[*ry, inca-12.5*] construct, which is sufficient for full phenotypic rescue of *inca* mutations, terminates between the end of the presented open reading frame and the polyadenlyation consensus. This indicates that the sequence in Figure 3.6 contains the essential *inca* coding region. However, it is not likely that this sequence presents the correct wild-type gene structure, because of the discrepancy between the predicted transcript size (4.2 kb) and the size of the band in Northern analysis (6 kb). We tested whether the genomic interval corresponded to sequences retained in mature transcripts by RT-PCR analysis. Antisense primers in the predicted 3' region were used to generate single strand cDNA from embryonic message, and the cDNAs were amplified using the polymerase chain reaction. All primer combinations were tested in control reactions using genomic DNA as the template. These experiments confirmed that sequences extending to position 3193 are utilized in mature transcripts, however, RT-PCR using primers at positions 3138 and 3872 did not give an amplification product. Notably, between these positions there are sequences corresponding to consensus splice donor sites. This suggests the existence of one or more additional exons 3' of the presented sequence; such are being sought in ongoing efforts.

To address the question of *inca* protein localization, as well as size and potential modifications, we generated two kinds of antisera. First, a synthetic peptide corresponding to the predicted mature N-terminus (exposed following cleavage of the signal sequence) was used to inoculate rabbits. Second, a construct representing 1.5 kb of exon 3, fused in frame to a poly-histidine epitope tag to facilitate purification, was expressed in bacteria to generate antigen for mouse injections. These sera will be referred to as N-*inca* and *inca*-fus, respectively.

inca expression in development.

Embryonic expression was examined by *in situ* transcript localization and by using N-*inca* antisera to stain wild-type animals. These two procedures yielded similar patterns. *inca* is abundant in newly deposited eggs, presumably reflecting maternal expression. Transcript and protein become ventrally localized and thereafter are widespread in mesodermal and some endodermal tissues, and in a subset of cell bodies in the nervous system (Figure 3.8A, B).

Antisera against *inca*-fus detects the same initial pattern, but in older embryos, staining is also found in axons and at surfaces of cells along the gut

(Figure 3.8C, D). Axonal detection is sharply contrasted with the cell soma localization of N-*inca* staining. We expect from this observation that cleavage of the amino terminus is part of the normal maturation of *inca* protein, and that mature processed protein lacks not only the N-terminal signal sequence but the subterminal region as well. An alternative explanation is that the protein takes on different conformations in different parts of the cell, and that in axons the protein is folded such that the amino terminus is inaccessible to antibody detection. *inca*^{1} mutant embryos show only background staining with both antisera, supporting the probability that both sera recognize *inca* gene products.

Although *inca* mutant embryos have defective tracheal morphology, *inca* transcript and protein are not found in tracheal cells. Rather, the gene is expressed in regions of the embryo that fail to become tracheated in *inca* mutants. This suggests that *inca* protein in the environment may act to induce or stabilize tracheal branching, or influence tracheal cell migration. The *breathless* mutant also shows aberrant tracheal morphology. This gene encodes an FGF receptor homolog that is expressed on tracheal cells (Klämbt et al., 1992). Although *inca* does not resemble FGF, it could act as an extracellular factor that affects tracheal development.

post-embryonic *inca* expression.

Larval and pupal tissues were examined for the localization of *inca* transcript and protein in imaginal visual cells. Transcript expression in the visual system during third instar is most apparent in the eye disc, posterior to the morphogenetic furrow. Antisera against *inca*-fus also detects protein in cells posterior to the furrow in the larval eye disc, as well as in photoreceptor projections across the optic stalk. No endogenous expression is found in the lamina at this time. Staining in the central brain and ventral nerve cord is reminiscent of the expression pattern in embryos. This stage also affords the best histological demonstration of vesicular localization of immature *inca* protein. The so-called giant nuclei, a cluster of neurons in the central brain, provide a particularly good example.

During pupal development, N-*inca* antisera shows widespread staining. Essentially all the cells in the head, including all the cells of the visual system, express the *inca* gene (Figure 3.9A). The protein is abundant in the vicinity of cell nuclei, but is not nuclear (Figure 3.9C). Some cells show staining in cytoplasmic projections, but never far from the nucleus. As is the case for

embryos, *inca*-fus antisera robustly stains axons (Figure 3.9B). The difference between the staining patterns with these two antisera is again consistent with a model of maturation by proteolytic processing. The sub-cellular localization provides an explanation for phenotypes observed in *inca* mosaic animals: protein synthesized in retinal neurons would be transported to the lamina neuropil region, and permitting uptake by lamina neurons. Note that this mechanism does not require that *inca* protein be incorporated into synaptic vesicles *per se*. Antibody staining indicates that maximal protein levels are reached in late pupal stage, consistent with Northern analysis of the *inca* transcript. Persistent taining is detected at a lower level after eclosion.

Discussion

The appropriate development of the *Drosophila* visual system depends on the ability of cells to interact both with their near neighbors and, via neuronal projections, with cells in different ganglia. Within the eye disc, inductive interactions in the specification of retinal cell fates have been extensively studied (Hafen and Basler, 1991; Ready, 1989; Rubin, 1989; Tomlinson, 1988). Several molecules that participate in signalling between different kinds of retinal cells have been identified (Cagan and Ready, 1989; Gaul et al., 1992; Heberlein et al., 1991; Krämer et al., 1991; Mlodzik et al., 1990). In contrast, interactions between the retina and the optic lobe are understood in much less detail. Although it is clear that retinal neurons regulate the development of their targets in the lamina (Selleck and Steller, 1991; Winberg et al., 1992), the molecular nature of this regulation is unknown. Contact between the retina and the brain is important for the survival of cells on both sides, but again no molecules have been described that affect trans-ganglion survival interactions in this system.

In this paper we present the initial characterization of a genetic locus that appears to be essential for both anterograde and retrograde maintenance signals between the retina and the optic ganglia. The *incomunicada* protein is required in photoreceptor neurons in order to promote target survival. This is the first report of the loss of fully differentiated lamina neurons in the presence of living photoreceptor cells. As such, if introduces the feasibility of studying neurotrophic interactions in the *Drosophila* visual system, a highly tractable genetic and molecular experimental regime. Perhaps more importantly, the

incomunicada protein itself may play a direct role in this interaction. Because inca acts non-cell-autonomously and is present in the extracellular environment, it may potentially be a ligand in a trophic signalling mechanism.

One concern at this point in that neuronal death in inca visual system mosaics occurs late in adult life, especially with regard to the time of peak expression of inca transcript and protein. Does this argue that inca is not directly responsible? We would suggest not, for several reasons. First, our assay for absent neurons is extremely crude and detects only the end stage of phenotypic manifestation. As methods for detecting early phases of cell death in Drosophila become more readily available, it may become evident that the "doomed" neurons are already aberrant in early adult life. Second, although we have demonstrated the existence of a trophic interaction, we have not determined when trophic dependencies arise, nor whether another trophic mechanism is active (and sufficient) during early adult stages. Finally, although peak inca expression occurs during pupal development, small amounts of protein are still present well into adult stage; low-level expression is a hallmark of trophic factors. Therefore, while the slow death timecourse should not be ignored, we feel that it does not contradict the possibility that inca plays a direct role in mediating a transneuronal trophic signal.

What is the *incomunicada* gene product? Gene sequence predicts, and sub-cellular localization suggests, that at least part of the *inca* protein is transported out of the cells that synthesize it. The *inca* precursor protein is apparently post-translationally modified during vesicular transport. Proteolytic processing is important in the maturation of numerous signalling molecules, many of which play essential roles both in development and in the maintenance of differentiated cells. It is interesting to speculate that *inca* may be a trophic factor itself, though no sequence homologies have been noted. In particular, a deficit of cysteine residues distinguishes *inca* from "typical" growth and trophic factors.

Though we have not noted any fate alterations in *inca* mutants, aberrant morphology of embryonic trachea could be explained by a failure of either an inductive or a trophic mechanism. Trachea are derived from the dorsolateral ectoderm, and extend branches centrally and ventrally well after gastrulation. We are particularly intrigued that the tracheal branches in *inca* mutants are most strikingly defective in the ventral and visceral regions of the embryo, where *inca* expression is normally highest. It should be noted that strong alleles of the

mutations *twist* and *snail*, which result in complete absence of mesoderm, also prevent tracheal development, though other dorsal ectodermal structures may be present (Simpson, 1983).

Eye-autonomous retinal degeneration mutants in flies have been described previously. With respect to lamina integrity, these mutations can be divided into two classes. An example of the first class is *retinal degeneration B (rdgB)*. Mutations in this gene lead to degeneration of both the retina and the lamina, with structural changes detectable within three days of eclosion. Interestingly, both ganglia are protected from degeneration if *rdgB* flies are raised and maintained in the dark, which presumably decreases synaptic activity (Stark et al., 1983). An example of the second class is *no receptor potential A (norpA)*. Severe alleles of *norpA* experience light-independent retinal degeneration, which progresses over several days in adult animals (Zinkl et al., 1990). This has not been previously correlated with an effect in the optic lobes, although we have observed late-onset lamina degeneration in $norpA^{P24}$ flies (not shown). The fact that the lamina is still vulnerable to deafferentation so late in the life of the fly indicates that target maintenance is a continuous process. Comparing the phenotypes of *rdgB* and *norpA* leads us to speculate that lamina degeneration arises by two different mechanisms. Rapid degeneration may result from active poisoning or excitotoxic effects, whereas late-onset degeneration following the removal of photoreceptor cells may reflect the loss of a trophic factor that is normally produced in the retina. Because *inca* clones in the visual system take so long to show degenerative effects, we expect that *inca* affects activity-independent signalling between neurons.

What are the consequences for photoreceptor neurons when cells in their target field are eliminated? In many experimental systems, input neurons that are deprived of their target tissues show dramatic degeneration. In some characterized cases, this target-dependent death is due to the loss of specific trophic factors (Levi-Montalcini, 1987; Oppenheim, 1991). Target ablation is often achieved by surgical means, not distinguishing between neuronal and non-neuronal cells in the target field. In *Drosophila*, retinal neurons that are unable to reach optic lobe targets eventually degenerate, but it is not known which cells in the brain are responsible for preventing retinal degeneration (Campos et al., 1992). Neurons and glia of both the lamina (the target field for the R1-6 class of photoreceptors) and the medulla (to which R7 and R8 photoreceptors project) could all potentially play a role. In the present analysis,

we found several examples of animals missing small numbers of either neuronal or glial cells in the lamina. In no case were these small areas of defective tissue correlated with degeneration in the retina. Others have previously reported the insensitivity of photoreceptors to the loss of a small number of lamina neurons (Brandstätter et al., 1992). These observations may indicate that the proposed trophic signal is diffusible, or that multiple cell types contribute to an essential trophic interaction. In a similar situation, the mosaic analysis of the brain degeneration mutant *drop-dead*, no phenotype was observed unless the brain was bilaterally mutant. This suggests that the remaining functional cells in the brain would produce an activity to rescue the mutant portions, even in the opposite brain hemisphere (Buchanan and Benzer, 1993; Hotta and Benzer, 1972).

One of the many remaining questions is the apparent contradiction between the non-cell autonomous activity of *inca* in retinal neurons, and the autonomous requirement for *inca* in glial cells of the lamina. One hypothesis is that these cell types process *inca* gene products differently, enabling the same gene to produce different functional activities. In support of this is the observation that one allele, $inca^{VE811}$, which produces an aberrant form of the *inca* protein, leads to glial death in the lamina, but gives no phenotype in retinal clones (see Figure 2d). Additionally, *inca* may be produced in a particular glial cell in order to affect its neighboring glial cell. Perhaps adjacent cells signal one another to carry out a coordinated process; cells that fail to produce the signal would not be included in the appropriate differentiation of the tissue.

Neural degeneration features prominently in pathology. Experiments aimed at reducing degenerative effects of neural lesions have concentrated on soluble factors. Already, such factors are being used to protect neurons against degeneration resulting from ischemia (Shigeno et al., 1991), hypoglycemia (Cheng and Mattson, 1991), and glutamate toxicity (Mattson et al., 1989). By studying the proteins that are responsible for neuronal maintenance in an experimental organism such as *Drosophila*, it may be possible to learn about cellular and molecular mechanisms that affect neuronal survival in more complex animals. For example, previous authors have noted molecular parallels between retinal degeneration mutations in flies and humans (Buchanan and Benzer, 1993). *Drosophila* embryos contain NGF-like activity (Hayashi et al., 1992), and homologs of neurotrophin and growth factor receptors have been described (Klämbt et al., 1992; Wadsworth et al., 1985;

Wilson et al., 1993). Interestingly, *inca* transcript shows specific hybridization to genomic Southerns of a variety of metazoans (not shown). Thus, *inca* homologs may exist that are required for maintenance of neural tissues in other organisms.

Experimental Procedures

Fly stocks. Standard methods were used for maintaining fly cultures. To generate lines for use in mosaic analysis, mutants were crossed to *y, w^{67c23}* and *y+, w* recombinants were tested for lethality. The enhancer trap line P1296 has the lacZ insert in polytene band 3D (Blair, 1992); it was obtained from the *Drosophila* Stock Center in Bloomington, along with Tp(1;3)*sta* and Dp(1;f)*101*. The Df(1)*su(s)^{83}*; Dp(1;3)*E2* strain was contributed from the laboratory of K. White, and lethal lines from the 1E-F region were obtained from N. Perrimon.

A screen for genes expressed in the visual system. New enhancer trap strains were generated by mobilizing the PZ[*lacZ, ry+*] element (Mlodzik and Hiromi, 1992) with the stably inserted transposase Δ2-3(99B) (Laski et al., 1986; Robertson et al., 1988) in the following cross: PZ, Cyo/+; Δ2-3(99B), Sb/ry^{506} X ry^{506}/ry^{506}. Cy+, Sb+, ry+ offspring were collected and backcrossed to ry^{506} to establish lines; these were screened for *lacZ* expression in the developing adult visual system of dissected third instar larvae (Chadwick, Berthon, Benson, Ressler, and Steller, unpublished).

Excision mutagenesis of the IV27b locus. Females homozygous for the X-linked IV27b element were crossed to Δ2-3(99B), Sb/ry^{506}; male progeny were crossed en masse to FM6/FM7; ry^{506} virgins, and *ry*, Sb+ daughters were collected. These were individually backcrossed to FM6; ry^{506} males to establish stocks. Stocks failing to produce non-balancer males were designated lethal. 472 lines were established; 440 were homozygous viable. These were examined for externally visible defects, especially in the compound eye; selected lines were also examined in frozen sections. No defects were detected in any of the viable revertant strains. 32 lethal lines were obtained; these were tested by crossing Δ27b/FM6; ry^{506} females to Tp(1;3)*sta* males. 5 lines produced sons with the genotype Δ27b; Dp(1;3)*sta*/ry^{506}, indicating that

the lethal locus on the X chromosome was contained within the duplication. These males were crossed to other mutant/FM6; *ry*^{506} lines and the crosses were scored for non-balancer, *ry* daughters, indicating complementation. Excision mutants were crossed to additional strains with duplications in the region; the smallest genetic interval was defined by the breakpoints of Dp(1;3)*sta* in 1E2 and Dp(1;f)*RA* in 1E4-5. Representatives of previously identified lethal complementation groups were similarly tested.

Cloning and molecular analysis. Standard molecular biological techniques were used (Sambrook et al., 1989). Genomic DNA flanking the IV27b insert was recovered by plasmid rescue (Steller and Pirrotta, 1986). Additional clones were obtained by screening Canton S genomic libraries in EMBL4, λdashII (R. Davis) and CoSpeR (J. Tamkun). DNA fragments of interest were subcloned into pBluescript KS+ (Stratagene) and sequenced by the chain-termination method (Sanger et al., 1977) using Sequenase (USBiochemicals). The sequence was analysed using GCG software (Genetics Computer, Inc.) and homology searches were performed using the BLAST program (Altschul et al., 1990). Primers 5'-TCGCTTGGCTTCTGTGTCG-3' and 5'-AGATAGATAGCGGACTCGG-3' were used in 5' primer extension reactions using 12 μg total embryo RNA per reaction. Primers were synthesized on an ABI PCR-mate DNA synthesizer.

Germline transformation of embryos. The 12.5 kb insert was recovered from phage #12 of the *inca* chromosomal walk, using λdashII vector-derived NotI sites; this was inserted into the pDM30 plasmid vector, which contains the wild-type *rosy* gene and a NotI cloning site between P element ends (Mismer and Rubin, 1987). This construct was co-injected with a transposase-encoding plasmid, pP25.7wc (Karess and Rubin, 1984), into 300 *ry*^{506} embryos prior to pole cell formation (<30 min after egg laying). Thirty-four adults were recovered and individually backcrossed to *ry*^{506}; from one of these crosses 2 ry^{+} flies were obtained, presumably representing products of the same germ cell transformation event. The construct is inserted on chromosome III.

Non-radioactive in situ mRNA localization was performed approximately according to Tautz and Pfeifle (1989), as adapted for RNA probes by R. Bodmer. Probe was made by *in vitro* transcription of the 2.1 kb *inca* cDNA in either sense

or antisense orientation, using T7 RNA polymerase and digoxigenin-dUTP (Boehringer Mannheim). Average probe length was reduced by carbonate treatment (100mM, pH 10.2). Embryos were collected and dechorionated, permeabilized with heptane, fixed 20 min with 4% paraformaldehyde in phosphate buffer (0.1M phosphate pH 7.2), methanol cracked, transitioned into ethanol, cleared with xylene and rinsed in ethanol followed by methanol. They were refixed with 50% methanol: 50% PBT plus 4% paraformaldehyde (PBT is phosphate buffer plus 0.1% Tween-20), and washed twice in PBT. Samples were then transitioned into hybridization solution and pre-incubated 1 hr at 55°C; boiled probe was added and incubation continued 18 hr. Hybridization solution consisted of 50% formamide, 5X SSC, 100μg/ml denatured herring sperm DNA, 100μg/ml yeast tRNA, 50μg/ml heparin and 0.1% Tween-20. Washes and detection were essentially as described (Tautz and Pfeifle, 1989).

To localize mRNA in larval tissues, washed larvae were dissected in 8% paraformaldehyde and tissues allowed to fix 1 hr on ice. Fixed tissues were washed in methanol plus 10% 0.5M EGTA, rinsed in ethanol, incubated in 50:50 ethanol:xylene 30 min, and rinsed several times with ethanol followed by methanol. Tissues were refixed in 50% methanol: 50% PBT plus 5% paraformaldehyde for 5 min, and then 25 min in 50% PBT plus 5% paraformaldehyde. After several washes in PBT, tissues were digested 3-5 min with 40 μg/ml Proteinase K (Sigma). Digestion was stopped with 2 mg/ml glycine in PBT, and tissues were refixed in 4% paraformaldehyde. Hybridization and washes were performed as for embryos above.

Generating and analysing visual system mosaics. Mutant alleles were recombined onto chromosomes bearing the w^{67c23} mutation, which acts cell-autonomously and results in white eyes. Females carrying *inca,* w^{67c23} chromosomes were crossed to males carrying either a dominant miniwhite construct inserted in polytene band 10 (Xu and Rubin, 1993), or a LacZ genotypic reporter, P1296, inserted in polytene band 3D (Blair, 1992). Embryos were collected for 12 hr, aged 24 hr and X-irradiated with 1500 rad to induce somatic recombination (Zusman et al., 1990), then allowed to continue development to adult stage. Adult heads were sectioned on a Reichert Jung Frigocut cryostat and antibody stained as described (Mismer and Rubin, 1987; Winberg et al., 1992). Alternatively, heads were processed (Campos et al., 1992) for embedment in plastic resin (Spurr, 1969) (Polysciences, Inc.) and 2

um sections were cut on an LKB microtome. Frozen sections were counterstained with 1ug/ml bisbenzimide (Sigma). Thin sections were stained with toluidene/methylene blue/borax at 70°C (Tix and Technau, 1989).

Antibody production. A 1.5 kb BamHI fragment from the *inca* cDNA, including 18 bases of polylinker from pBluescript KS+, was subcloned into the pET15b vector (Novagen) to produce a protein with a 6-His tag fused to 482 amino acids coded by exon 3 of *inca*. The protein was insoluble when expressed in BL21-DE3 bacteria, therefore the pellet was dissolved in 8M urea. Solubilized denatured protein was purified on a Ni-NTA column (Qiagen) according to manufacturer's instructions. Eluted fractions were inspected for protein bands near 55 kD. Positive fractions were pooled and run on a preparative SDS-PAGE gel; the major band was excised, electro-eluted and dialyzed against PBS. Purified protein was mixed with MPL+TDM emulsion (RIBI ImmunoChem Research, Inc.) to give a final concentration of 0.1 mg/ml; 0.2 ml of emulsion was used for each immunization and boost for each mouse. Additionally, a synthetic peptide, LPTIQGGNARQQLDKC, corresponding to the predicted N-terminus of *inca* pro-protein plus a cysteine residue, was coupled to KLH using SMCC. This was mixed with RIBI MPL+TDM+CWS emulsion to give a final concentration of 0.1 mg/ml; 1 ml of emulsion was used for each immunization and boost for each rabbit. Animals were injected at intervals of approximately 4 weeks for mice and 6 weeks for rabbits; in all cases usable antisera were obtained after the third injection.

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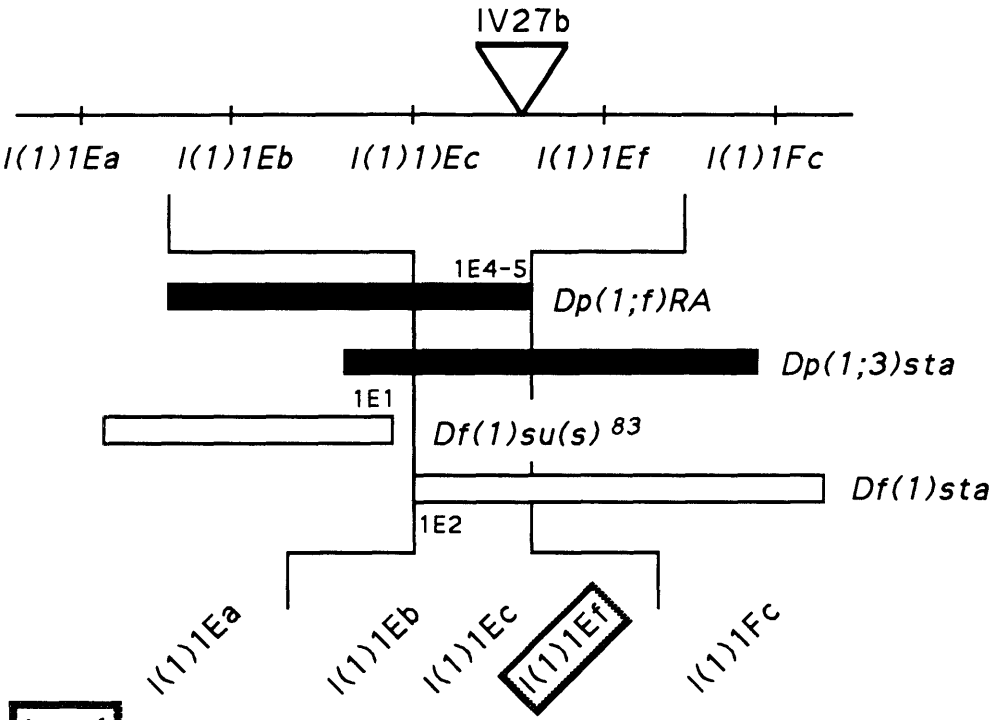
Figure 3.1. Expression of IV27b in the developing visual system.

Horizontal frozen sections of pupal heads stained for the IV27b reporter, which contains a nuclear localization signal. (A) Activity staining for beta-galactosidase detects nuclei of photoreceptor neurons (arrows) and essentially all the cells of the lamina. Glial cells surrounding the medulla neuropil and a few other cells in the medulla cortex are also stained. (B) Counterstaining with bis-benzimide highlights cells which don't express the reporter. la, lamina; me, medulla; re, retina.

Figure 3.2. Genetic analysis mutations in the IV27b region.

The site of insertion for IV27b was mapped to polytene chromosome band 1E on the X chromosome. At the top is a schematic representation of identified nearby lethal complementation groups in linear order, relative to the insert. Center: Overlapping duplications define the limits of a genetic interval for mutations in the IV27b locus between 1E2 and 1E4-5. Filled boxes indicate duplications and open boxes indicate deletions. Bottom: Complementation analysis of excision mutants: *inca*^{1} failed to complement l(1)1Ef. Three lethal mutants, *10.28Δ27b, 10.29Δ27b* and *11.13Δ27b*, failed to complement l(1)1Ec. The *16.28Δ27b* excision failed to complement two loci and was not used in subsequent studies. Cytology is as given in Lindsley and Zimm (1992), Perrimon et al., (1989), and Belyaeva et al., (1982).



	l(1)1Ea	l(1)1Eb	l(1)1Ec	l(1)1Ef	l(1)1Fc
inca 1	+	+	+	−	+
16.28	+	+	−	−	+
10.28	+	+	−	+	+
10.29	+	+	−	+	+
11.13	+	+	−	+	+

Figure 3.3. Phenotypes in adults mosaic for *inca*.
Semi-thin plastic sections of adult heads stained with toluidine and methylene for contrast are depicted in all panels except (E), which is a frozen section stained with anti-betagalactosidase. (A) Section through one half of a wild type head, showing characteristically well-ordered arrangements of cells. (B) A clone of *inca*^{1} mutant cells in the retina of a young adult fly is identified by the loss of pigment granules. The arrangement of ommatidial cells is essentially the same as in pigmented regions, and the underlying optic ganglia appear normal. (C) A retinal clone in an animal which was aged 21 days post eclosion. Cells within the *inca*^{1} mutant patch appear normal, but the region of the lamina which is innervated by mutant cells lacks neurons. Glial nuclei are still present within the neuropil of the affected region of the lamina (arrows). The medulla is not significantly affected at this level of resolution. (D) A retinal clone of *inca*^{VE811}, aged 61 days post eclosion. No degeneration is detectable. (E) Frozen section of a young fly mosaic for *inca*^{VE811}. Glial nuclei are detected within the lamina neuropil (closed arrows); some glia are missing from the center (open arrows). All of the nuclei which are present are LacZ^{+}, indicating that they are wild type for *inca*. We interpret that lamina glia are missing due to a loss of *inca* function in the glia themselves. (F-K) Semi-thin sections of flies mosaic for *inca*^{1}. (F) Missing glial cells. Normally the cytoplasm of glial cells in the neuropil is darkly stained by toluidine (closed arrowheads) and the absence of cells is easily detected (open arrows). Lamina cartridges retain near-normal structure despite missing glia. Note that this preparation is not stained for beta-galactosidase. (G and H) Examples of missing glial cells at the lamina neuropil boundaries, resulting in holes in the tissue. (I-J) Frontal sections of an aged mosaic fly with degeneration in the retina and lamina. The retina was genotypically wild type. Genotypes of internal cells could not be determined. The retina and lamina are severely affected; the outer medulla is abnormal, possibly due to the loss of afferent fibers. (J) Close view of the retina and lamina: some rhabdomeres are still present, but many ommatidia lack dark rhadbomere staining and neuronal nuclei are absent or misplaced. Remnants of glial cytoplasm in the lamina are seen. (K) Unaffected contralateral eye of the same animal. Note neuronal cell bodies positioned between intact rhabdomeres (arrows). (L) Section of an aged *inca*^{1} mutant fly which carries one copy of the *P[ry, inca-12.5]* transgene. No degeneration is evident in either the lamina or the retina.

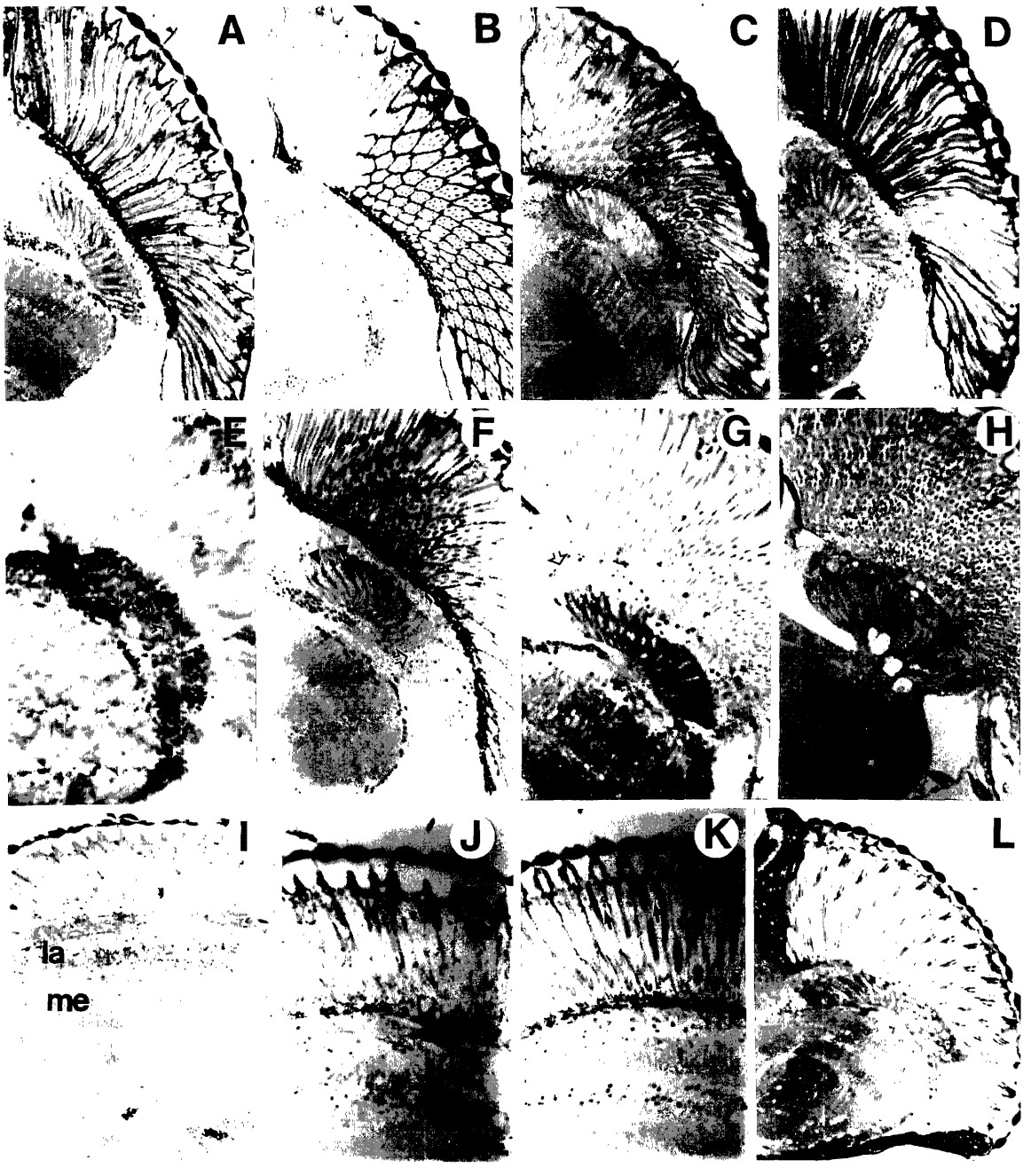

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Figure 3.4. Tracheal morphology of *incomunicada* mutants.

(A) Monoclonal antibody staining 2A12 of a wildtype embryo soon after inter-segmental tracheal fusion. The antigen is readily detected in the dorsal longitudinal trunk and less obvious in the ventral and visceral branches. Arrows indicate ventral tracheal branches which have just begun to label. (B) In a mutant embryo of the same age, the antibody detects only a single longitudinal trunk. (C) An 18-hr wildtype embryo shows extensive tracheal branching; the optical quality of the network is altered by filling with air. (D) A mutant embryo of approximately the same age. The unbranched, unfilled dorsal trunk is indicated between arrows. The dark structure is a malphigian tubule. All panels are lateral views, anterior left and dorsal up.

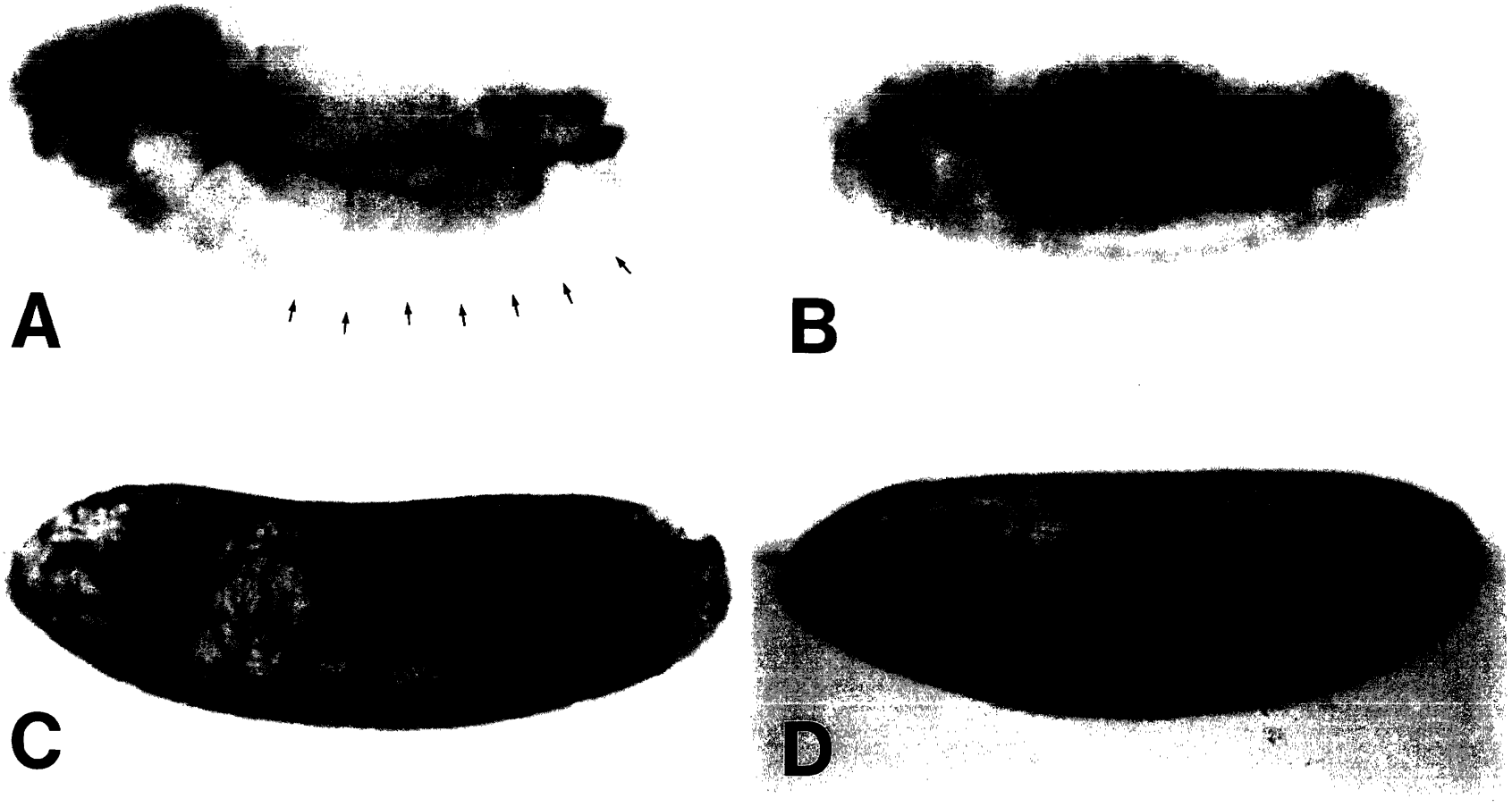
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Figure 3.5. Structure of the *incomunicada* locus.

The site of insertion of the IV27b P element is indicated by a triangle. The horizontal line represents DNA which was cloned in a phage walk that started from the P element; HindIII and EcoRI sites are shown. Open boxes below the line indicate the extent of DNA deleted in $inca^{1}$ and $inca^{C24}$ mutants, the hatched box shows the approximate location of the $inca^{GA91}$ translocation breakpoint. Two excision mutations, *2.15Δ27b* and *11.13Δ27b*, are also indicated at left; these deletions complement all the *inca* alleles in the present study. The *inca* transcript is depicted at top center; the arrow shows the direction of transcription and the filled regions indicate translated sequence. The HindIII fragment which was used to probe for transcripts is indicated by an asterisk. The only other known transcript in the region is shown at top right, with the direction of transcription indicated by the arrow. The filled box at the bottom represents the genomic region used for germline transformation; this construct rescues all *inca* phenotypes which have been identified (see Figure 3).

incomunicada

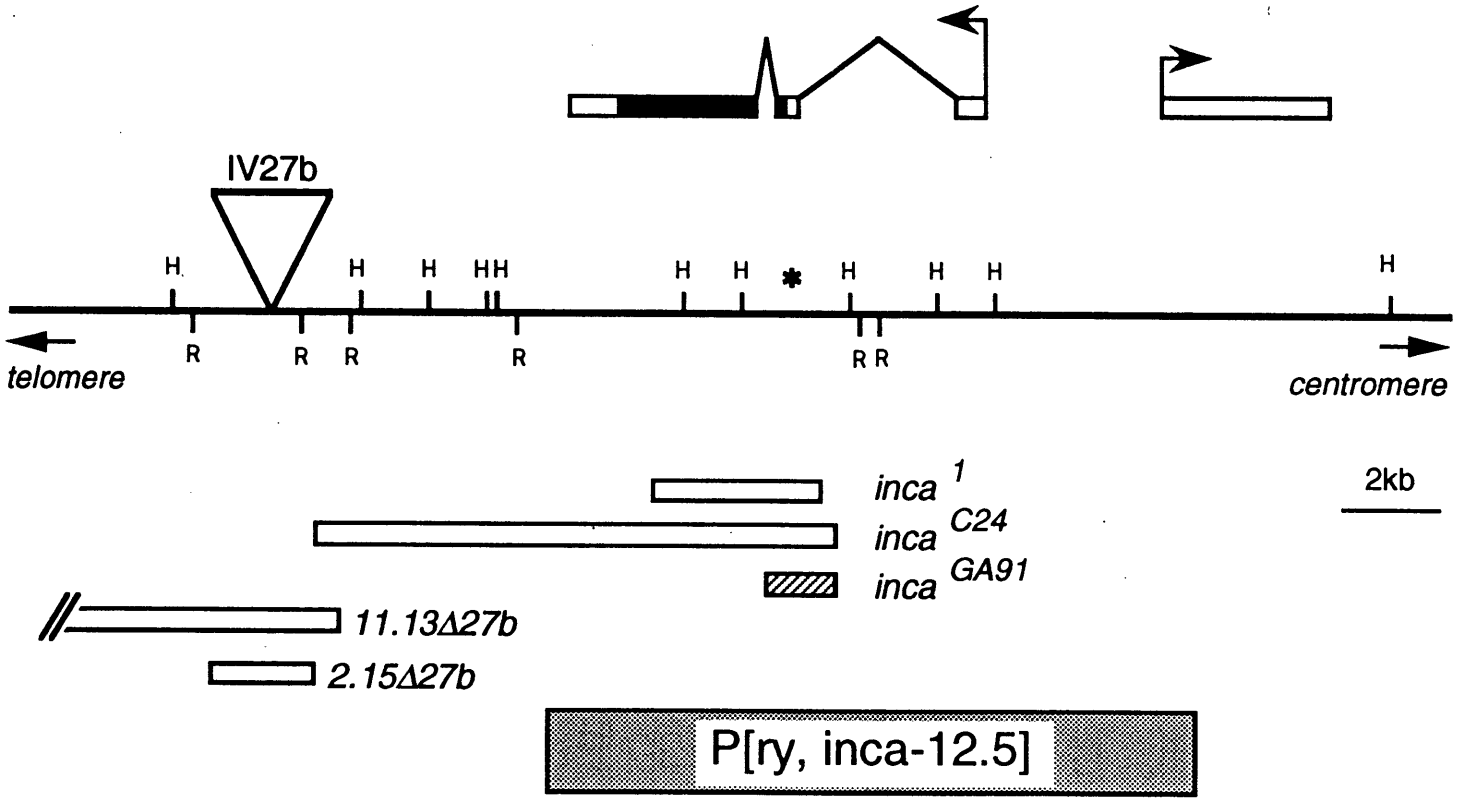
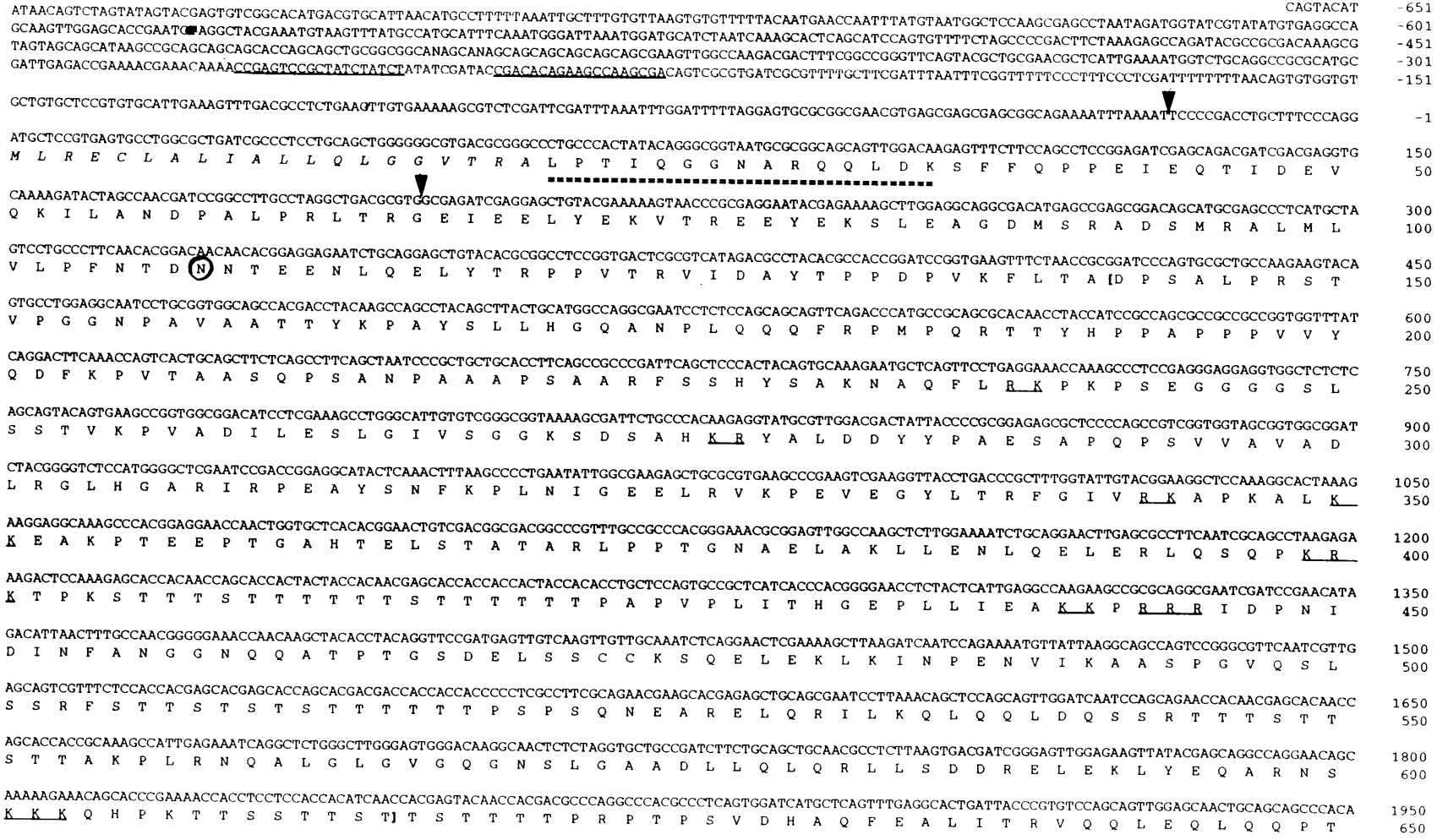


Figure 3.6. Nucleotide and protein sequence of *incomunicada*.

The figure represents a composite of sequences obtained from cDNA and genomic clones. Numbering is relative to the predicted translation initiation site. (Other initiation codons lie upstream of this position but they are followed by very short ORFs.) The *inca* cDNA clone extends from -321 to +1841 in the DNA sequence. The positions of 2 introns are indicated by arrowheads; these were found by comparing the cDNA with genomic sequence. Italicized residues at the N-terminus indicate a probable signal sequence which would be removed from the mature protein. Doublets and triplets of basic residues are underlined; in some proteins these sequences are used as the sites of proteolytic processing. Sites of potential N-linked glycosylation are indicated by circled residues. The N-*inca* antisera was generated against the double-underlined peptide sequence. Brackets indicate the region included in the *inca*-fus construct (140 to 615 in the protein sequence). The downstream bracket also indicates the end of the cDNA clone. The 3' end of inca transcript is predicted from genomic sequence: the reading frame remains open for almost 2 kb past the end of the cDNA clone. Consensus polyadenylation sequences are found approximately 340 and 485 bases downstream of the amber stop codon. The 5' end of the transcript was subjected to primer extension analysis, using oligonucleotides complementary to the underlined sequences in the 5' untranslated region. Primer extension yielded short products consistent with premature termination at GC-rich regions approximately 140 and 190 bases upstream of primer 1, and an additional product of about 300 nucleotides in length. In view of CAAT and TATA sequences further upstream, this raises the possibility that transcription may be initiated at approximately -560 bases on the coordinates shown.



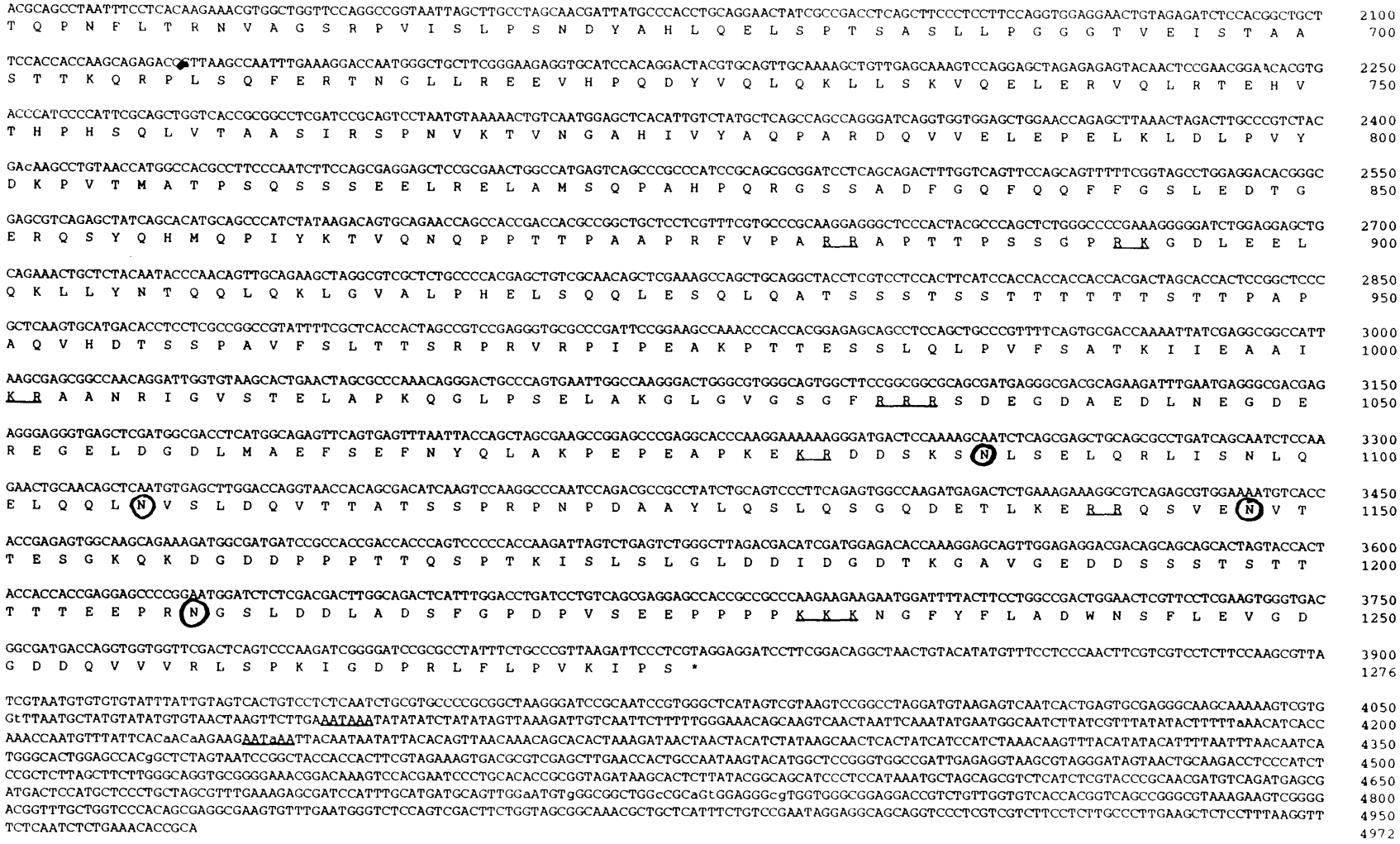
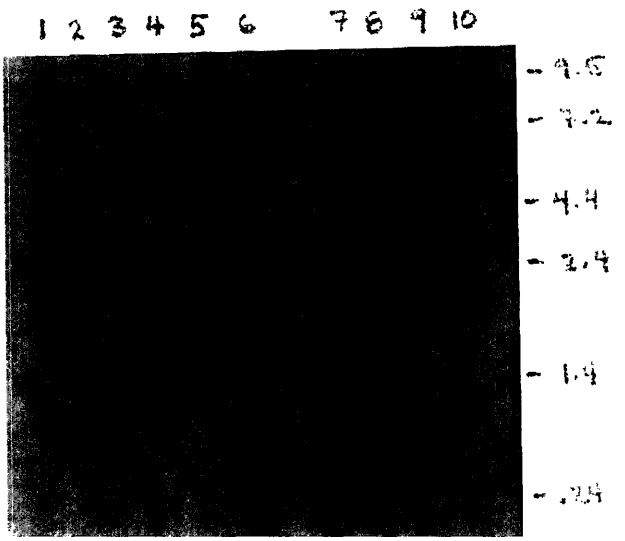


Figure 3.7. Northern analysis of the *incomunicada* locus.

A 1.5 kb fragment from exon 3 was nick-translated and used to probe poly-A^+ RNA from a series of developmental stages. A single band of about 6 kb is detected, and is most abundant in late pupal stage. Lane 1, ovary; 2, 0-2 hrs AEL; 3, 2-5 hrs; 4, 5-8 hrs; 5, 8-24 hrs; 6, young larvae; 7, old larvae; 8, young pupae; 9, old pupae; 10, adult.

Figure 3.8. Embryonic expression of *incomunicada.*

inca gene products were localized by in situ hybridization to mRNA (A-C) or immunohistochemical detection with inca-fus antisera (D-F). Protein and transcript are uniformly distributed in the oocyte and become localized to the ventral half of the embryo by blastoderm stage. Inca is abundant in invaginating cells of the mesoderm (A) and in mesodermal and endodermal tissues during gastrulation (B). By mid-embryogenesis, expression is most apparent in the gut and the central nervous system, including a segmentally repeated pattern in the ventral nerve cord (C). Expression in a few cells in the periphery is obscured by the strong visceral signal. In older embryos, protein is localized along surfaces of the gut and peripheral sense organs (D), in sensory axons (E), and in a subset of axons in the CNS (F). All embryos are depicted with anterior to the left. Panels A, B and C are lateral views with ventral down; D is a high magnification ventral view.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9.5
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1.4
.28

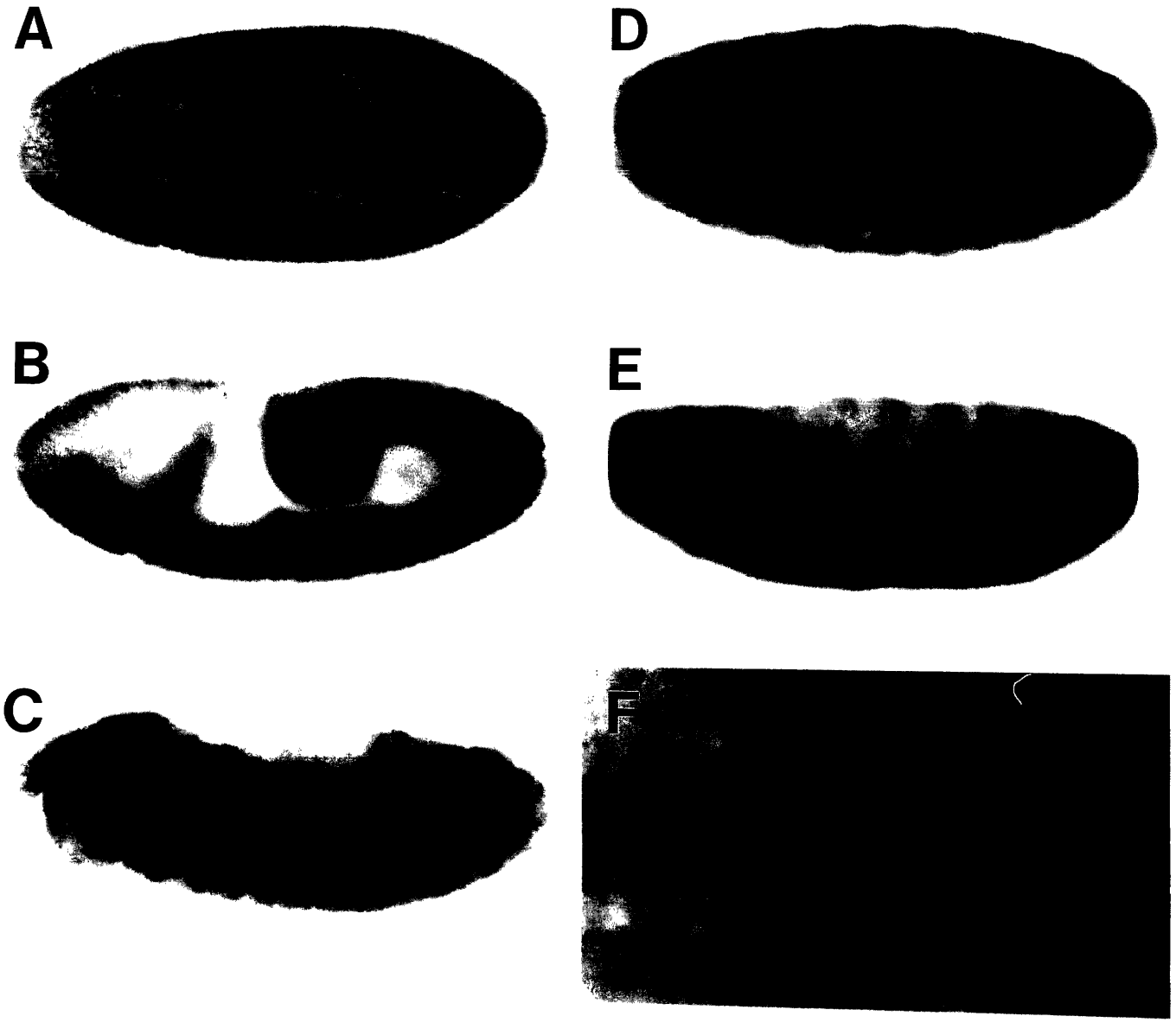
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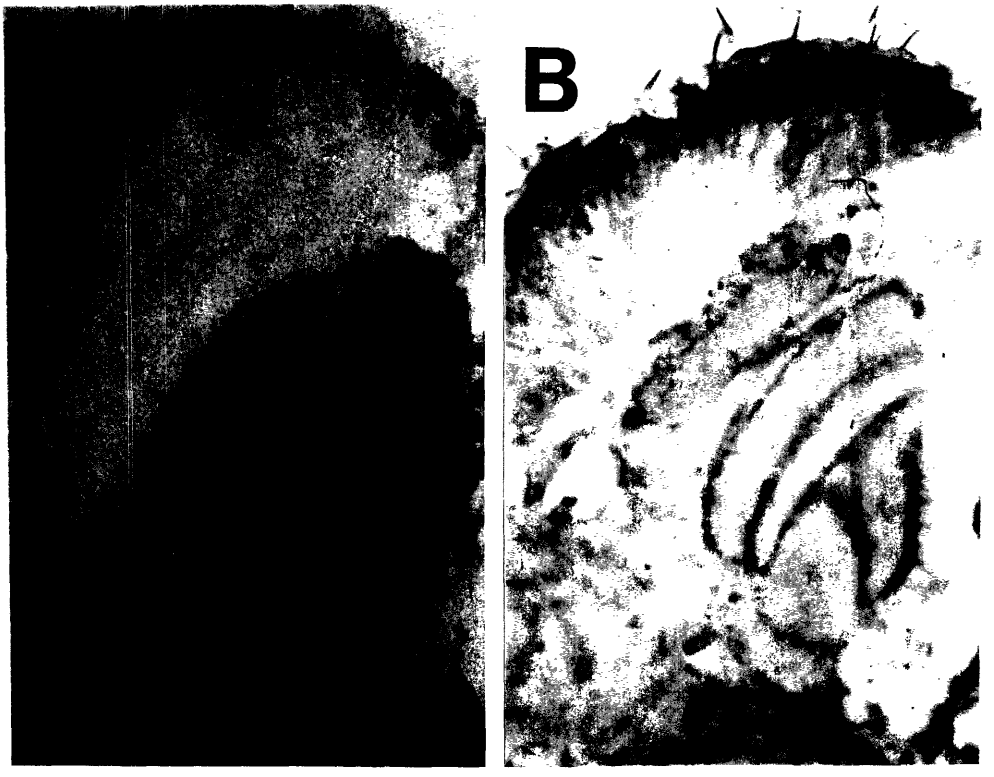
Figure 3.9. Expression of *incomunicada* in the visual system.

Antibody staining of horizontal frozen sections during pupal development. (A) N-*inca* antisera stains nearly all cell bodies in the head. (B) An adjacent section from the same animal, stained with *inca*-fus antisera, shows staining throughout the retina, and axonal projections in the optic lobe. (C) High magnification view of N-*inca* staining in the brain shows a pattern of sub-cellular localization suggestive of secretory vesicles.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Chapter IV. Concluding remarks.

A graduate student's work is never done- though sometimes it must be taken up by someone else for continuation. This chapter is an attempt to give some perspectives on the results in this thesis, to point out hidden assumptions about the work, and to suggest some directions future investigations could take.

Assembling the first optic ganglion.

The experiments in the second chapter were performed with a certain amount of anticipation. We expected from our reading of the literature that glial cells or their immediate precursors must already reside in the lamina prior to photoreceptor axon ingrowth. Yet, we also knew there must be a mechanism for regulating glial differentiation and survival, in order to achieve correct geometry in the mature lamina. Thus the demonstration of photoreceptor-dependent transcriptional induction, while a useful advance, was not really a surprise. Presumably a large number of genes should be required to complete glial differentiation, and it is aesthetically sensible that these genes should be inactive until photoreceptor axons grow into the optic lobe.

Less expected were the results of the lineage tracing experiments. Despite historical biases about the distinction between neurons and glia, in most cases these differentiated cell types come from the same kinds of precursor cells, and it is possible to find small clonal populations of mixed cell types. We approached these studies with the model of the *Drosophila* retina in mind: the parallel posterior-to-anterior development of the retina and lamina, and the similarities between ommatidial and cartridge structures lead one to speculate that they would be assembled according to similar rules. Therefore we guessed that clones of cells in the lamina would fall roughly according to cartridge shapes. This was in fact true if one looked only at neuronal cells: clonal boundaries within the neuronal layers were consistent with the expectation that neurons in a cartridge are more likely than not to arise from the same precursor, or at least closely related cells. In contrast, clonal boundaries usually separated glia completely away from neurons in the same cartridge, indicating that these cell types are derived from distinct lineages. In fact, our data are consistent with the possibility that glial precursors and neuronal precursors in the optic lobe may be derived from cells which are already distinctly specified at the time they are incorporated into the optic lobe anlage in mid-embryogenesis. If so, it is possible that markers indicating differential gene

expression within this group of cells will also reveal this distinction. Equally intriguing was the separation of glial subtypes within the lamina, correlating specific glial functions and morphologies with specific lineages.

These lineage tracing experiments, performed by following the loss of a somatic marker, posed two immediate questions that would be better addressed with a different system of marking clones. First, is there a strict correspondence between neuronal lineage and incorporation into a cartridge? This question can be broken into several bites. Are the last two mitotic products of a ganglion mother cell always found in the same cartridge? If so are they always adjacent, like the pairs of cells in an 8-member tetrad? During third instar, lamina neurons are found in columns of "about 8" cells, rather than the clusters of 5 found in adults. If one examined 8-cell clones in larvae, would all 8 be in the same column? Or would they occupy homologous positions in, say, 2 adjacent columns? Or would they be more random? Incidentally, these questions also bear on the mechanism of cells crossing the lamina furrow, a structure that is superficially similar but physically quite distinct from the morphogenetic furrow of the retina. The retinal furrow forms as a consequence of cell shape changes associated with synchronous cell divisions in a monolayer epithelium. In contrast, the lamina furrow resembles a tube in horizontal sections; the cells are displaced rather than simply contracted. If 8-cell clones in the lamina show an ordered arrangement, then this implies some coherence of clonally related cells in their passage across the furrow.

The second question posed by the lamina lineage tracing experiments regards the physical location of lamina glial precursor cells. One might expect that lamina glial precursors should reside parallel, but perhaps medial, to the lamina neuronal precursors that come from the outer proliferation center. This would put glia in a good position to respond to the same posterior-to-anterior inductive wave as do neuronal precursors. However, some of the clones we observed in the lineage trace suggested that anterior lamina neurons are closely related to anterior external medulla neurons. If this observation is extrapolated a bit, one can imagine that neuroblasts in each part of the outer proliferation center generate two kinds of cells: medial cells that are later incorporated into the medulla and lateral cells that become incorporated into the lamina. Therefore if lamina glial precursors arise medial to lamina neuronal precursors, then nascent medulla neurons or ganglion mother cells would be forced to migrate between the lamina glia to get to their appropriate positions.

Nothing in the literature suggests that this is the case, though admittedly nothing in the literature really addresses the problem.

The questions raised in the last two paragraphs can be addressed more easily using a positive lineage marker rather than the loss of a marker. While retroviral infection has not been much used in *Drosophila*, several investigators have had success with an inducible lineage tracer. The utility of the tracer stems from the adoption of the site-specific yeast FLP recombinase system: a LacZ reporter is positioned between two FLP recombinase targets (FRTs) in reverse orientation to a strong promoter. At the desired time in development, the recombinase is induced, for example by heat shock, and cells in the appropriate part of the cell cycle can "flip" the reporter into the active configuration. The system offers the investigator substantial control over the size and location of clones, depending on previous knowledge of cell proliferation patterns. This system could be used to generate small clones of marked lamina neurons by inducing in late third instar, or to generate large clones of lamina glia through earlier induction. Large glial clones would in some cases span from positions within the lamina, where glial cells can be unambiguously identified, to positions where glial precursors reside. Et voilà!

Lamina glial cells and photoreceptor axon guidance.

Underlying all this interest in the behavior of lamina glial cells in third instar is the assumption that these cells contribute in some way to the navigation of photoreceptor growth cones. This assumption is based entirely on physical proximity, though much effort is being made to find experimental evidence for it. Current approaches follow two general plans: enhancer trap screens for genes that are either specific to one or another glial type or that show graded polar expression, and direct screens for mutations that confer axon-guidance defects on third instar photoreceptors. Enhancer trapping is a two-edged sword: while it enables rapid molecular progress on the selected genomic locus, it provides no guarantee of functional significance, and it has the potential to lead the investigator a long way from the intended goal. And especially in the present question, the critical molecules may be expressed in a precursor population whose position is unknown! These problems are circumvented by following a mutagenesis scheme. However, while direct screens are sure to lead to the correct phenotype, screening in third instar is

tedious and potentially eliminates the examination of axon guidance molecules that are required in embryonic development.

A third approach, again with its own bias, would be to collect those embryonic lethal mutations that have axon guidance defects, and test them for visual system expression and phenotypes in mosaic animals. This fairly derivative plan would be a poor way to spend one's career, but could provide an appropriate supplement to screens for new axon guidance molecules. At the very least, it holds out the possibility of rapid demonstration of the premise!

Characterization of the *incomunicada* gene and protein.

We have presented *inca* as a candidate effector molecule in cell-cell interactions. To further the case for this argument, an appropriate next step would be to get (or make) a full-length cDNA and move into a cell culture system. By expressing large quantities of wild-type *inca* in a homogeneous culture, several questions could be addressed. First, is *inca* really secreted, or does it stay attached to the membrane somehow? Second, is the same protein species really recognized by the two antisera? This could be addressed using immunoprecipitation followed by Western, a technique that is would be inefficient (but not impossible) starting with primary tissue. Third, is *inca* constitutively processed into the same proteolytic fragments as seen *in vivo*, or is there evidence for a regulated proteolytic pathway? Fourth, what part of the protein is represented in various pieces (i.e. what are the N-terminal sequences of the protein fragments)? Answers to these questions could be coordinated with a genetic engineering approach to *inca* function: how much of the *inca* gene must be reintroduced by germline transformation to rescue the lethal phenotype? Can different protein domains be separated into functional units that would be used by different cell types? What are the fates of the various pieces? The last question could be easier to answer using epitope-tagged versions of the protein.

With regard to *inca* function, I have held the assumption that the critical part of the protein is in the N-terminal half. This is based on the observations that (1) epitopes recognized by the *inca*-fus antisera are retained in axons and (2) the rescue construct P[*ry, inca*-12.5] probably lacks the 3' end of the gene. Of course, this is also based on ignorance of the real C-terminus, so future investigators should beware!

incomunicada in other organisms.

A low-stringency "zoo blot" using the *inca* 2.1 kb cDNA as a probe detected single bands in a variety of species, most robustly in *Drosophila virilis* and less so in crustaceans, nematodes and fish. To find *inca* homologs in other organisms, it would be effective to first screen for the *D. virilis* gene by standard hybridization methods. This species is removed from *D. melanogaster* by 60 million years of evolution, which should make it similar enough to be easily cloned. Whatever divergence has occurred should highlight those parts of the sequence most likely to be retained through greater evolutionary times. Additional homologs in other species could then be sought by either hybridization or PCR screens. The most immediate utility of this series of experiments is to identify conserved regions of the gene, those areas most likely to make the greatest contribution to structure and function. This evolutionary approach has the potential to progress more rapidly than the culture and transformation experiments outlined above, and is somewhat more likely to generate outside interest. Over the long term, if a mammalian *inca* homolog would play a role in neuronal cell viability in people, then it may eventually become important in diagnostics or treatment of some nervous system disorder.

Phenotypes in *Drosophila*.

The phenotypes described for *inca* mutants and mosaics in the previous chapter raise a large number of interesting questions, many of which can be addressed using available reagents and technology.

The principal tracheal defect in older *inca* embryos is a lack of lateral branches. How does this come about? In the normal developmental pathway for tracheal formation, dorsal, ventral and visceral branches form in each segment prior to fusion of the longitudinal trunk. Are these branches present early in *inca* mutants and then lost, or do they never form? Is there insufficient tracheal cell proliferation? Are the correct number of cells present, but misplaced (possibly all adopting the trunk configuration)? Tracheal cells are not easily seen in light microscopic preparations until such time as the lumens fill with air, but these questions could be addressed utilizing a tracheal specific marker of reasonable quality. My attempts to label immature trachea with monoclonal antibody D3 have been less than clearly interpretable. Obtaining a

suitable enhancer trap line, preferably with a nuclear signal and low background, should facilitate matters.

With regard to phenotypes in visual system mosaics, several questions and experiments come to mind. For example, when does the lamina begin to show the effects of retinal *inca* deprivation? In plastic sections, lamina neurons appear to be intact and healthy for at least 7 days after eclosion, and dead at 20 days. My efforts to narrow this window were unfortunately done using the $inca^{VE811}$ allele, which later was found not to manifest this phenotype at all. These experiments should be repeated with either $inca^{1}$ or $inca^{C24}$. Another possibility would be to look for an earlier defect than actual death, say a morphological change, or onset of expression of a "cell death gene". In conjunction with mosaic analysis, it would be very helpful to create a situation in which the entire retina would be mutant, especially if the lamina retained wild-type expression. There are several ways in which this might be achieved. First, one could use an unstable X chromosome to generate mosaics. At some low frequency, the appropriate conditions would be met. Second, one could engineer a retina-specific antisense *inca* construct, perhaps driven by the *glass* or *chaoptic* promoter. Third, one could try to engineer or screen for a new loss-of-function allele of *inca* that would be viable but show visual defects: the inverse version of the $inca^{VE811}$ allele. (Here is one strategy that is not difficult, but it could take a lot of screening: mutagenize males, cross them to $inca^{1}$/FM7, and score aged non-balancer F1 daughters for aberrant visual behavior and/or retinal degeneration. This kind of screen could also potentially isolate glial-specific or brain-specific alleles. For example, lack of *inca* function in the lamina glia could lead to cell death, thereby disrupting electrical isolation of the lamina and leading to abnormal electroretinograms, if not also to defective organismal behavior, as in the *reverse potential* mutation.) I assume that if the lamina degenerates due to lack of *inca* function in the retina, then the desired flies would go blind over time. It would also be very interesting to know the electrophysiological consequences of lack of *inca* function in the retina. Perhaps the electrical behavior of the retina and lamina is normal prior to the onset of degenerative changes, but it may also be that electroretinograms or intracellular recordings of photoreceptors or lamina neurons would find an earlier abnormality. Future investigators should know before they begin, however, that the X chromosome has been pretty closely checked for visual system structure and behavior mutations, and those screens did not find *inca*.

Granted, this may well be due to irrelevant technical constraints- very few people wait until their flies are 3 weeks old before they look at them!

Another big question concerns the requirement for *inca* function in lamina glia. We are sure that these cells must express *inca* in order to "make it" to adult stage, and I have assumed that because *inca* is not detected in the lamina of third instar, it must not be required until after pupariation. This blatantly disregards the possibility that *inca* is expressed or required in optic lobe precursors. This can be tested through more detailed expression analysis in embryonic and larval development (using antibodies rather than *in situ* hybridization), or by looking for living mutant glia in visual system mosaics during late larval and early pupal stages. The latter approach is more technically difficult, but more informative, because it also provides insight as to the time course of glial cell death. This in turn pertains to the question of whether *inca* is required for differentiation or for survival, again assuming that it is not required for glial cell generation.

The analysis of brain mosaics in the previous chapter is not very satisfying, mostly due to the inutility of the reporter construct for determining cellular genotypes in aged animals. The search for reliable, ubiquitous X-linked genetic markers that are readily detected in older animals has drawn a complete blank. Although such a reagent still has not been found for general applications, the N-*inca* antisera should be suitable for this investigation. The mosaic analysis could be repeated using frozen sections and antibody staining, but to look at fine structure, it might be worthwhile to investigate either water-soluble plastic embedding (permitting antibody staining after sectioning) or whole mount antibody staining of heads prior to embedding in plastic or paraffin. Reliable genotyping would allow much more conclusive statements about the prospect of retinal degeneration due to lack of *inca* function in the brain. The previous effort could not definitively assert that retinal degeneration was not due to X-rays. For this reason, it would be useful to generate some mosaics through a different protocol: perhaps ring X, FLP, or transplantation.

With regard to retrograde interactions and the retina, I predict that the retinal degeneration phenotype will turn out to derive from defective lamina amacrine neurons, or be totally phony. My reasoning depends on the fact that in the few animals where retinal degeneration was found, it was spread all over the retina. Low frequency suggests that the offending cell type is not abundant (i.e. not hit very often). There are only 3-4 amacrine neurons in each lamina,

but they are highly branched, each one contributing to synapses throughout the lamina neuropil. One problem with this prediction is that R8 cells, which do not interact with amacrine neurons, do actually degenerate. A competing hypothesis points to neurons in the external medulla, and stipulates very large clones that would affect centrifugal projections across the entire visual field.

Other molecules in the pathway.

If *incomunicada* is a signal, then what are the other molecules involved in signal transduction? The search for interacting genes could go any number of ways. Direct molecular interactions between ligands and receptors have been the basis for many successful expression cloning projects using cultured CHO cells or Xenopus oocytes to express cDNAs. (I would think seriously about this one.) The recent development of "interaction trapping" in yeast looks very promising, though this approach may not be the best for extracellular molecules. Affinity column purification of interacting proteins is a method with many biochemically-minded fans. And then there's always fly genetics.

Gene-interaction screens to find enhancers or supressors in flies have worked very well for mutations that confer certain kinds of phenotypes: abnormal eye morphology, number of bristles, pigmentation changes, blindness, etc. They have not generally been tried starting with lethal alleles. To get around this, one can make a viable loss-of-function allele, or engineer a gain-of-function allele that overexpresses or ectopically expresses the starting protein. The new phenotype should be easily scored. For *incomunicada*, the best bet might be a viable loss-of-function allele that conferred a visual defect, as discussed above. Then an F1 suppressor screen could be carried out on mass populations. Alternatively, ectopic expression in embryos might lead to an easily scored defect (other than death) that could form the basis for an F2 screen. One of the advantages of the genetic approach is that it works to reveal genes both upstream and downstream in the pathway, and a successful screen yields several kinds of molecules. It also avoids the biochemical complications of heteromultimeric receptors and low affinity interactions.

Finally, due to the recent blossoming of interest in tracheal development in the Krasnow lab, it may be that they are already working on a gene with the same phenotype as *inca* embryos. Perhaps the fastest way to get interacting genes is by phone!

Appendix: Preliminary characterization of *l(1)1Ec*

Introduction

The enhancer trap screening method in *Drosophila* has provided an effective tool for identifying essential genes that affect post-embryonic development. It has also provided the opportunity to induce mutations near the site of P element insertion in interesting strains, via imprecise excisions. Although such mutations are somewhat directed, they are not entirely specific. As a result, excision mutagenesis quite frequently presents the investigator with multiple complementation groups to analyze. In generating excision mutations around the IV27b P element, I recovered mutations in two adjacent lethal complementation groups. One of these was described in Chapter III. This appendix gives a brief analysis of mutants in the second complementation group, called *l(1)1Ec*.

Results

The enhancer trap line IV27b, inserted in polytene band 1E4, was identified in a screen for lines expressing LacZ in the developing visual system. To initiate a molecular and genetic study of loci near the P insert, genomic DNA flanking the insertion was recovered by plasmid rescue (Steller and Pirrotta, 1986). A chromosomal walk was performed in phage and cosmid libraries (R. Davis and J. Tamkun). At the same time, excision mutagenesis was undertaken, as described in Chapter III. Of all the excision strains that were recovered, only 5 showed a mutant phenotype (lethality) that mapped near the insertion site of IV27b. One of these strains corresponded to the *l(1)1Ef* complementation group and is now called *incomunicada*. Another excision strain, *16.28Δ27b*, affected two complementation groups; it has not been used in subsequent studies. Three of the excision mutants had apparently single-gene mutations in the *l(1)1Ec* complementation group; these are *10.28*, *10.29*, and *11.13* (Table A.1). This complementation group was previously reported to have a larval lethal phase, but has not been characterized in any detail (Perrimon et al., 1989).

Using DNA probes from the phage walk, genomic Southerns of the mutant lines were screened for rearrangements. Figure A.1 presents a map of the region and a summary of identified rearrangements in the cloned interval. The IV27b element is apparently inserted between two genetic loci, raising the question of which locus corresponds to IV27b. Mutant animals from both complementation groups were examined for structural abnormalities, but neither showed defects that were obviously related to the nervous system expression pattern of the P element reporter. Because my primary interest was in the visual system, it seemed best to perform mosaic analysis, testing for visual system phenotypes.

Based on the expression of the IV27b reporter, I expected that mutant retinal clones of the corresponding endogenous gene should show some kind of defect. Two alleles from each complementation group were recombined onto chromosomes bearing the *w*^{67c23} mutation, which was used to mark retinal mutant clones in a wild-type eye color background. Somatic mosaics were induced by X-ray irradiating larvae during the first instar. Heads of adults with retinal clones were processed for plastic sectioning and semithin sections were cut (Tix et al., 1989; Campos et al., 1992). Figure A.2 illustrates the phenotype of *l(1)1Ec* retinal clones: in the mutant area, the lenses are separated from the underlying tissue. In other respects, the ommatidia have an apparently normal configuration, showing the correct number of photoreceptors in the appropriate stereotyped array, and cone and pigment cells surround the neurons as usual. The altered morphology of the ommatidia does interfere somewhat with the identification of the primary pigment cells in the center of the clones, but these cells are readily found at the edges (see panel B). Facets in the mutant clones were not noticeably misshapen when examined under a dissecting microscope, suggesting that the clone started with the appropriate arrangement of cone cells to secrete the lens.

Separation of the lenses from the underlying cells could be caused by ectopic cell death at the lateral margin of the eye. However, examination of the affected tissue suggests that the appropriate cell types are still present; moreover, there is no evidence of dying cells in the clones (Abrams et al., 1993).

In the process of mapping breakpoints for the excision alleles, it became apparent that one of the mutants, *10.29*, had retained the LacZ gene from the original enhancer trap. This mutant was therefore examined for the expression

pattern of its reporter. The pattern differed substantially from that of IV27b, with the essential features as shown in Figure A.3. Reporter expression begins in the midgut very early in embryogenesis, and continues throughout larval development. In third instar, reporter activity is detected in both larval and imaginal cells of the gut. Also in third instar, strong expression is seen in photoreceptor neurons of the developing eye. The pattern of expression during pupal and adult stages has not been determined. In particular, it is not known whether the reporter is ever expressed in non-neuronal cells of the eye, which are affected in retinal clones.

Discussion

The phenotype displayed in retinal clones of *l(1)1Ec* is in some ways reminiscent of that reported for *l(1)myospheroid*, a *Drosophila* integrin. In their analysis of *l(1)mys*, Zusman et al., (1990) found clones in the eye that contained the appropriate cell types, but the retina was separated from the underlying lamina, leading to ommatidial disarray. Subsequent study indicated that *l(1)mys* is required late in pupal development, during the time when retinal pigment cells form the basal fenestrated layer (Zusman et al., 1993). It seems likely that the adhesive function of *l(1)mys* contributes to the formation of this structure. Without it, retinal cells lose their precise organization. In comparing these results to those shown above, it is tempting to speculate that the defect in *l(1)1Ec* clones is due to the failure of a second adhesive function in the pigment cells, in this case at the apical surface.

In addition, the embryonic, larval and imaginal midgut expression of the *10.29* reporter is highly reminiscent of another *Drosophila* integrin, β_V (Yee and Hynes, 1993). While this similarity may be simple coincidence, perhaps it reflects a meaningful correlation. Few *Drosophila* integrins have been found relative to the large number of them in vertebrates, and it is presumed that additional fly integrin genes exist. It is possible that *l(1)1Ec* encodes one of these, or that this gene contributes in some other way to an adhesive function in *Drosophila*.

Materials and Methods

See Chapter III for stock-keeping, enhancer trap screen, excision mutagenesis, molecular cloning and mosaic analysis methods.

Beta-galactosidase activity staining of dissected larval tissues was as described in Chapter II. For embryos, overnight egg collections were washed in water, dechorionated in 50% Chlorox, washed, transferred to heptane and fixed in 0.5% glutaraldehyde in PIPES buffer plus 10mM $MgSO_4$ for 15 minutes at room temperature, shaking. Embryos were then collected in the heptane phase and dropped onto double-stick tape on a Sylgard plate, quickly air dried and covered with PBS. The sharp end of a broken pulled Pasteur pipette was used to release embryos from their vitelline membranes, and the "peeled" embryos were then stained for beta-galactosidase as above.

Acknowledgements

I would like to thank G. Yee, K. Stark and R. Hynes for helpful discussion of the mutant phenotype and *10.29* expression pattern. K. Stark is continuing the analysis of *l(1)1Ec* in the laboratory of R. Hynes.

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Table A.1. Complementation Analysis. Excision alleles generated from the mobilization of the lV27b enhancer trap were initially tested by crossing to Tp(1;3)*sta*, a transposition that covers the original insert. Local lethal excisions were then tested against each other and against representatives of all known lethal complementation groups covered by the transposition. (See also Figure 3.2.) These stocks were obtained from N. Perrimon.

♀ ♂	3.12	10.28	10.29	11.13	16.28	synonym
3.12	-	+	+	+	-	inca[1]
10.28	+	NT	-	-	-	
10.29	+	NT	-	-	-	
11.13	+	-	-	-	+/-	
16.28	-	-	-	-	-	
EA48	+	NT	+	+	+	
VA209	+	NT	+	+	+	
A102	+	-	-	-	-	l(1)1Ec
C24	-	NT	+	+	-	l(1)1Ef
VE676	+	NT	+	+	+	
HC156	+	NT	+	+	+	
VA92	+	NT	+	+	+	
VA185	+	NT	+	+	+	
EA97	+	NT	+	+	+	
A70	+	NT	+	+	+	
VE676	+	NT	+	+	+	

Figure A.1. A chromosomal walk, with identified breakpoints of *l(1)1Ec* rearrangements. Two complementing deletions are included at left: *l(1)C24* and *2.15Δ27b*. pX27b indicates the plasmid rescue of the lV27b locus, cosT3A and cosT3B represent cosmid clones obtained from J. Tamkun's library in the CoSpeR vector, and the remaining clones indicated were derived from phage library screens. ϕ3 and ϕ5 are in the EMBL4 vector and the others are in λdashII. All of these libraries were made from Canton S flies. At the bottom, solid lines indicate DNA that is deleted in the various mutants, and hatched bars indicate areas of ambiguity. For example, the GA119 allele shows a rearrangement compared to Canton S that affects the 14 kb HindIII fragment but is distal to the 9 kb EcoRI fragment.

The three EMS-induced alleles have not been examined by Southern analysis.

The cosmid clones were isolated using the 2.7 kb EcoRI fragment as a probe. In the screen, the probe also hybridized to another group of cosmids that were clearly related to each other and not to the walk shown here. The probe cross-hybridizes to a 5 kb EcoRI fragment in the second group. No other "spurious" cosmids came through the screen. This may indicate the presence of a real gene in the fragment that was used as a probe, with a second member of a gene family in the other group of cosmids. Alternatively, it may be junk.

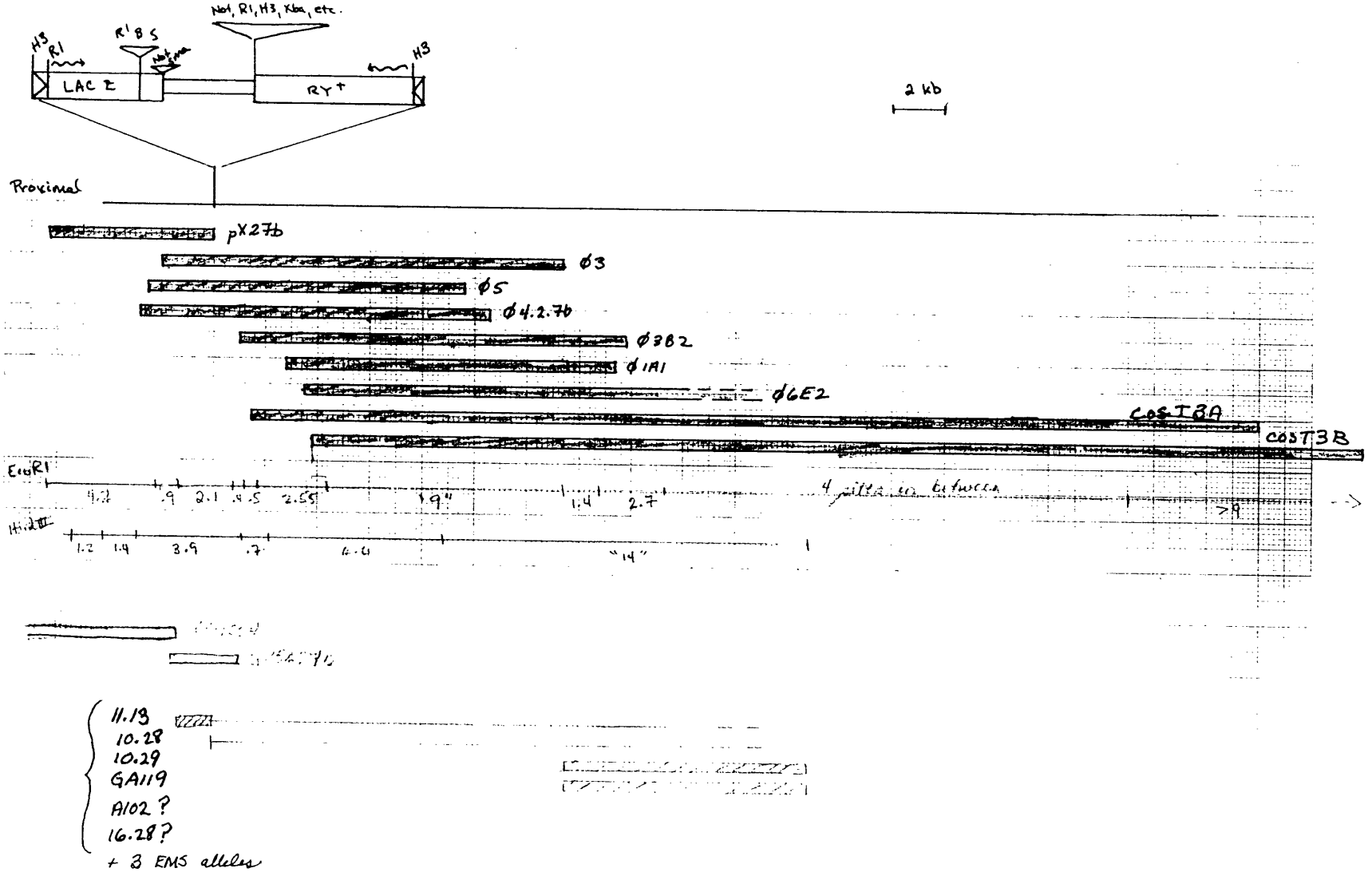

Not, R1, H3, Kba, etc.
H3
R1
R1 B S
LAC Z
RY+
H3
2 kb
Proximal
pX27b
ϕ3
ϕ5
ϕ4.2.7b
ϕ3B2
ϕ1A1
ϕ6E2
COS 1BA
COS 3B
EcoR1
4.2
.9
2.1
.4
.5
2.55
1.9"
1.4
2.7
4 sites in between
>9
H.2
1.2
1.4
3.9
.7
4.6
"14"
1
11.19
10.28
10.29
GA119
A102 ?
16.28 ?
+ 3 EMS alleles

Figure A.2. Clones of *l(1)1Ec*. Panel A, a clone of the *11.13* allele is marked by the lack of pigment granules. In the mutant region (between arrows) the lenses are separated from the underlying retinal cells. Panel B, closeup of a clone of the *10.28* allele suggests the normal complement and arrangement of cells (compare with Figure 1.2). The number and placement of rhabdomeres is the same in the pigmented and unpigmented regions. Secondary pigment cells are in place in the mutant region, even though they contain no pigment granules. The primary pigment cells, which normally form the "spike" between the cone cells and the lens, are defective, but appear to be present. This is most easily seen at the edge of a clone, where a wild-type primary pigment cell and a mutant primary pigment cell are in contact, and the spike reaches only partway to the lens.

This phenotype is manifest as soon as the flies eclose from the pupal case.

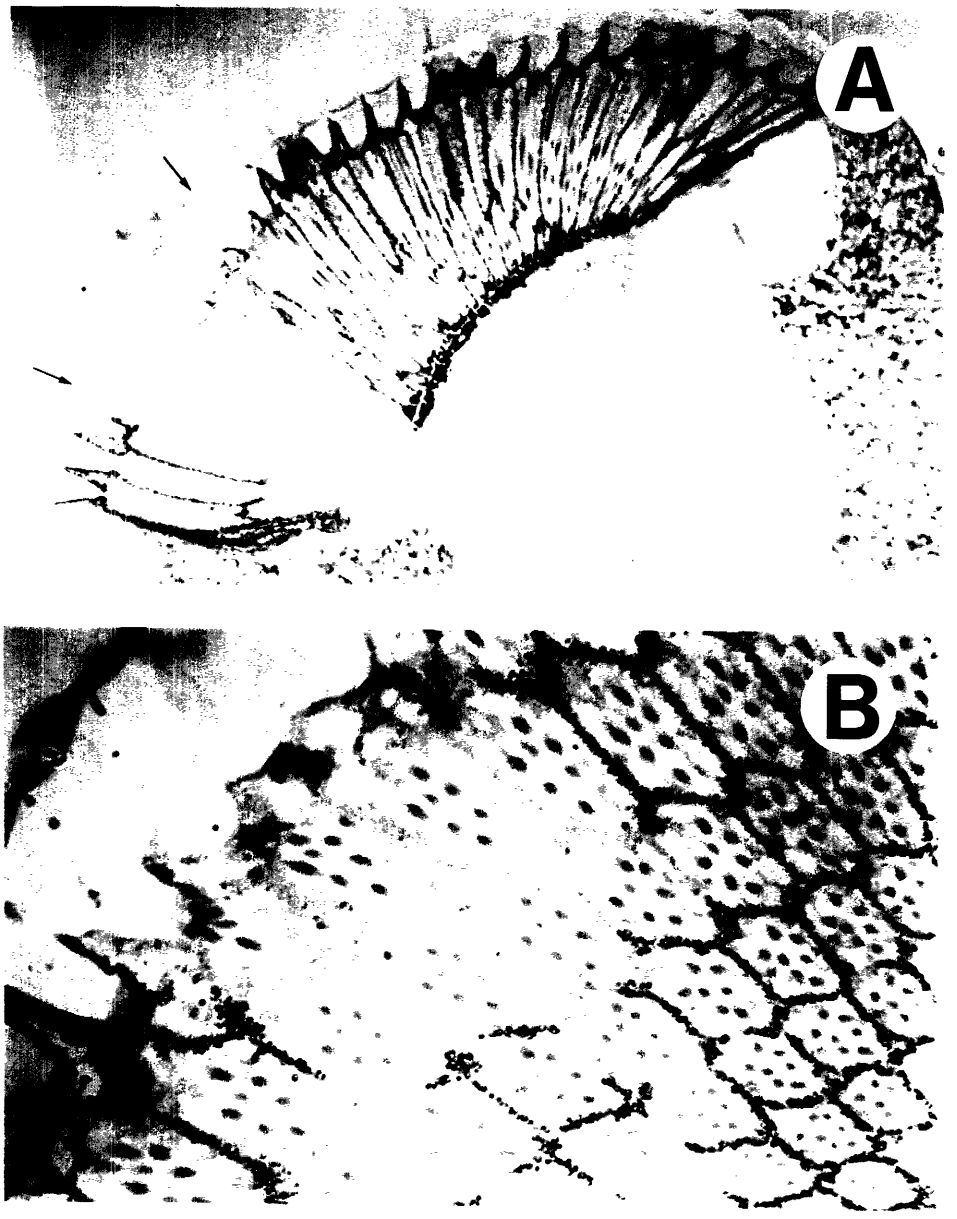
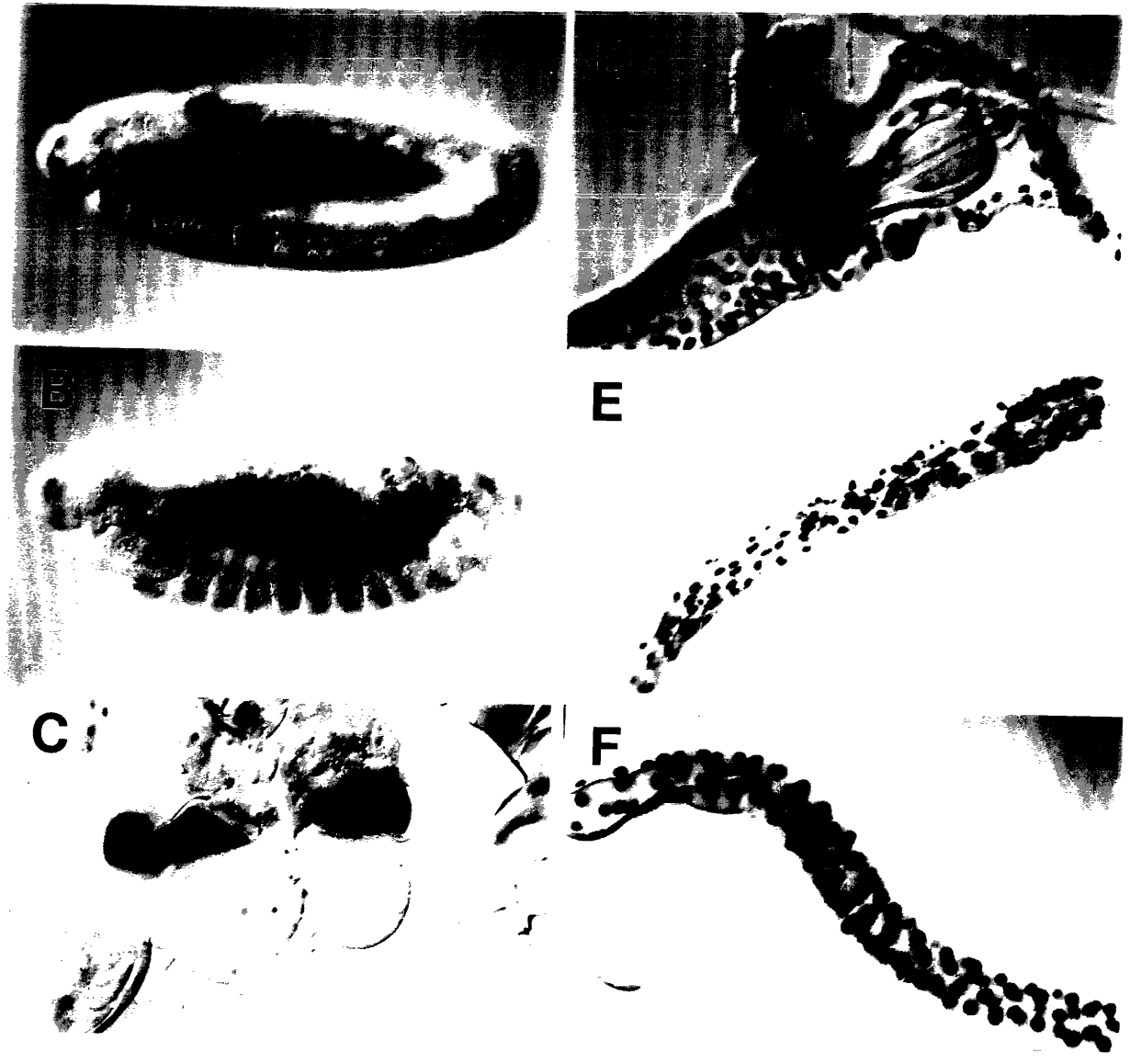

A
B

Figure A.3. Embryonic expression of beta-galactosidase in the *10.29* allele. Note that the reporter construct contains a nuclear localization signal. Panel A, darkest staining is in the anterior and posterior midgut primordia. Punctate staining in the dorsal anterior region is in cells of the amnioserosa. Panel B, in older embryos, most of the stain is in the midgut, with additional staining in a segmentally repeated pattern in unidentified cells in the periphery. Panel C, third instar larval brain and imaginal discs. The photoreceptors of the developing eye disc are strongly stained. Panel D, anterior midgut, proventriculus and gastric caeca. Panel E, large cells of the larval midgut and small imaginal islands that give rise to the adult digestive tract. Panel F, the larval salivary gland is stained and the associated fat body is not.


D
E
C
F