Ablation of *Drosophila* photoreceptor cells by conditional expression of a toxin gene

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We have used toxin-mediated ablation to study some aspects of visual system development in *Drosophila melanogaster*. To devise a method that permits the conditional expression of a cellular toxin, we introduced an amber mutation into the diphtheria toxin-A-chain gene. In transgenic animals, this toxin gene can be activated by providing the gene for an amber suppressor tRNA. By coupling this toxin gene to the photoreceptor cell-specific promoter of the *chaoptic* gene, photoreceptor cells could be specifically ablated during development. Photoreceptor cell-specific markers normally activated during pupal development failed to appear after midpupation. Photoreceptor cells were absent from the retinas of adult flies at eclosion. We have assessed the consequences of photoreceptor cell ablation for eye and optic lobe development. We suggest that the larval photoreceptor nerve is not essential, in the late larval stages, for retinula photoreceptor cell axons to achieve their proper projection pattern in the brain. Moreover, while retinula photoreceptor innervation is initially required for the development of normal optic ganglia, the ablation of these cells in midpupation has no discernible effect. This approach to cell-specific ablation should be generally applicable to the study of cellular functions in development and behavior.

[*Key Words*: Toxin-mediated ablation; *Drosophila*; photoreceptor cell development]

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The analysis of cell–cell interactions during development has been greatly aided by methods that permit the selective removal of cells of a particular identity. When the ablation of particular cells results in defined changes in fate among neighbors, one can infer a role for cell–cell communication in the determination of cell fate. For example, in the nematode, the use of laser microbeam ablation has been an especially powerful tool for identifying developmental lineages that are not rigidly determined (Sulston and White 1980; Kimble 1981). Laser ablation of cells of the grasshopper neural ectoderm has revealed the plasticity of the decision to adopt a neural fate (Doe and Goodman 1985).

Methods for selective cell ablation might also be applied to the study of visual system development in the fruitfly *Drosophila*. The development of the larval visual system begins in the embryo with the bilateral innervation of the brain optic lobe anlagen by the larval photoreceptor nerves (also called Bolwig's nerves; for review, see Meinertzhagen 1973). In the late larval stage, the developing adult visual system utilizes this axonal pathway for the projection of retinula photoreceptor cell axons into the brain (Trujillo-Cenóz and Melamed 1973). A key role for cell–cell interactions in the formation of optic ganglia has long been proposed on the basis of the optic lobe defects observed in mutants lacking proper innervation by photoreceptor cells (Power 1943; Meyerowitz and Kankel 1978; Fischbach 1983; Steller et al. 1987). Finally, specific cell–cell interactions are responsible for the determination of cell fate in the developing retina (for review, see Tomlinson 1988; Ready 1989; Rubin 1989). Additional details of these events may be revealed by ablating cells in a defined manner during development.

However, because of the small size and opaque cuticle in *Drosophila*, methods such as laser microbeam ablation have been difficult to apply. Furthermore, these methods do not permit the removal of large numbers of cells that are dispersed in position or in developmental time. Finally, such methods do not permit the convenient isolation of a population of individuals bearing identical ablations, which would facilitate genetic, biochemical, and behavioral analyses. A new approach to cell ablation, which has been successfully applied in the mouse, relies on the cell-specific expression of the gene for a cellular toxin (Palmiter et al. 1987; Landel et al. 1988; for review, see Evans 1989). By placing a toxin-coding sequence under the control of a cell-type-specific promoter, a defined set of cells can be specifically and reproducibly ablated during development. This strategy has been successful in ablating cells of the exocrine pancreas (Palmiter et al. 1987) and the lens of the eye (Breitman et al. 1987; Landel et al. 1988).

To apply toxin-mediated ablation in *Drosophila*, we

chose to utilize the diphtheria toxin-A-chain (DT-A) gene, which encodes the A polypeptide fragment of diphtheria toxin (Maxwell et al. 1986; Palmiter et al. 1987). The toxin-A fragment catalyzes the inactivation of the translational factor EF-2, resulting in the arrest of protein synthesis (for review, see Collier 1975; Pappenheimer 1977). The DT-A gene does not include the second subunit of the native protein that facilitates transit across cellular membranes (Uchida et al. 1973). Its action is therefore expected to be cell-autonomous.

To transform *Drosophila* with the appropriate DT-A gene construct, we found it necessary to place DT-A gene expression under conditional control. Our attempts to introduce the fully functional gene by germ-line transformation failed, apparently as a result of the very high lethality associated with microinjection of a DT-A gene construct. Similar observations have been made in other laboratories (see Discussion, below). By introducing an amber mutation into the toxin-coding sequence, its expression in vivo could be regulated by the presence or absence of an amber suppressor tRNA gene. We show that photoreceptor cells are specifically ablated during the pupal stage in transformants that express diphtheria toxin under the control of a photoreceptor cell-specific promoter. Finally, we have examined the structure of the adult optic ganglia in these transgenic lines to help define the role of photoreceptor cell innervation in optic ganglia development.

Results

Germ-line transformation with a DT-A gene construct

In several other laboratories, attempts to recover *Drosophila* germ-line transformants with constructs bearing the fully functional DT-A gene have been unsuccessful (see Discussion, below). We reasoned that the difficulty might be overcome by preventing even very low levels of transient toxin gene expression in microinjected embryos, as such expression might lack the appropriate tissue specificity (see, e.g., Steller and Pirrotta 1984). To place toxin synthesis under conditional control, we introduced an amber termination codon into the toxin-coding sequence. A plasmid containing the DT-A gene (Maxwell et al. 1986; Palmiter et al. 1987) was used as the substrate in a polymerase chain reaction with a mutant oligonucleotide primer (Fig. 1) .

To direct toxin expression to photoreceptor cells, we utilized the promoter region of the *chaoptic* (*chp*) gene, which is expressed only in photoreceptor cells (Zipursky et al. 1984). After approximately hour 12 of embryonic development, *chp* is expressed continuously in cells of the larval photoreceptor nerve. During the late third instar, *chp* is expressed in the retinula photoreceptor cells shortly after they begin to differentiate in the eye–imaginal disc. A large genomic DNA fragment containing 5′-untranslated sequences of the *chp* gene (Reinke et al. 1988), which confer photoreceptor-cell-specific expression upon a reporter *lacZ* gene (H. Steller and G. Rubin, unpubl.), was inserted upstream of either the wild-type or amber mutant DT-A-coding sequence in the *P*-element transformation vector pUChsneo (Fig. 1; Steller and Pirrotta 1985).

Germ-line transformation (Rubin and Spradling 1982) was performed with the wild-type and amber mutant toxin constructs. Microinjection with either of two independent DNA preparations of the wild-type toxin plasmid resulted in very poor survival; 94% of injected embryos failed to hatch. No transformants were recovered. By using instead the mutant toxin plasmid, the survival of injected embryos increased to ~50%. One germ-line transformant was subsequently recovered. This line is denoted *P*[DT-A, *neo*]12. Because flanking genomic sequences often influence the expression of transgenes in *Drosophila* (Spradling and Rubin 1983; Bourouis and Richards 1985; Wakimoto et al. 1986), the insert was mobilized by the introduction of a source of *P* transposase. Ten additional independent transgenic lines were recovered. In some cases, these lines are referred to by their insertion numbers (e.g., *P*[DT-A, *neo*]12 as line 12).

Head-specific expression of the toxin gene

To confirm the expected tissue-specific pattern of toxin gene expression, the transgenic lines were assayed for the presence of DT-A mRNA transcripts in separated adult heads and bodies. Because the eye constitutes a large portion of the adult head, DT-A mRNA should be relatively abundant in the head and absent from the body. For the two transgenic lines shown in Figure 2 , a DT-A DNA probe detects several mRNA species in the heads of freshly eclosed adults. No such mRNA species are detected in either the bodies of the same collection of individuals or in Canton-S (CS) flies. The head-specific expression of toxin mRNA was observed in all of six different lines tested (data not shown). Among the different lines, the size and amount of the toxin mRNA species varied (Fig. 2; data not shown). The size variation is likely due to the extension of transcripts into flanking genomic sequences resulting from the absence of a well-utilized polyadenylation signal in the *P*[DT-A, *neo*] element. The differing amounts of transcription product may be due to the influence of flanking genomic sequences on the rate of transcription or to differences in stability among the transcripts. Regardless, the presence of the DT-A transcripts in the expected tissue is consistent with their proper expression in photoreceptor cells. Furthermore, the presence of an appreciable level of these mutant transcripts in adult tissue suggests that they are tolerated for at least several days after the onset of expression.

Effects of toxin gene expression on the structure of adult eyes

To permit the translation of a functional DT-A gene product, the transgenic lines were crossed to a strain

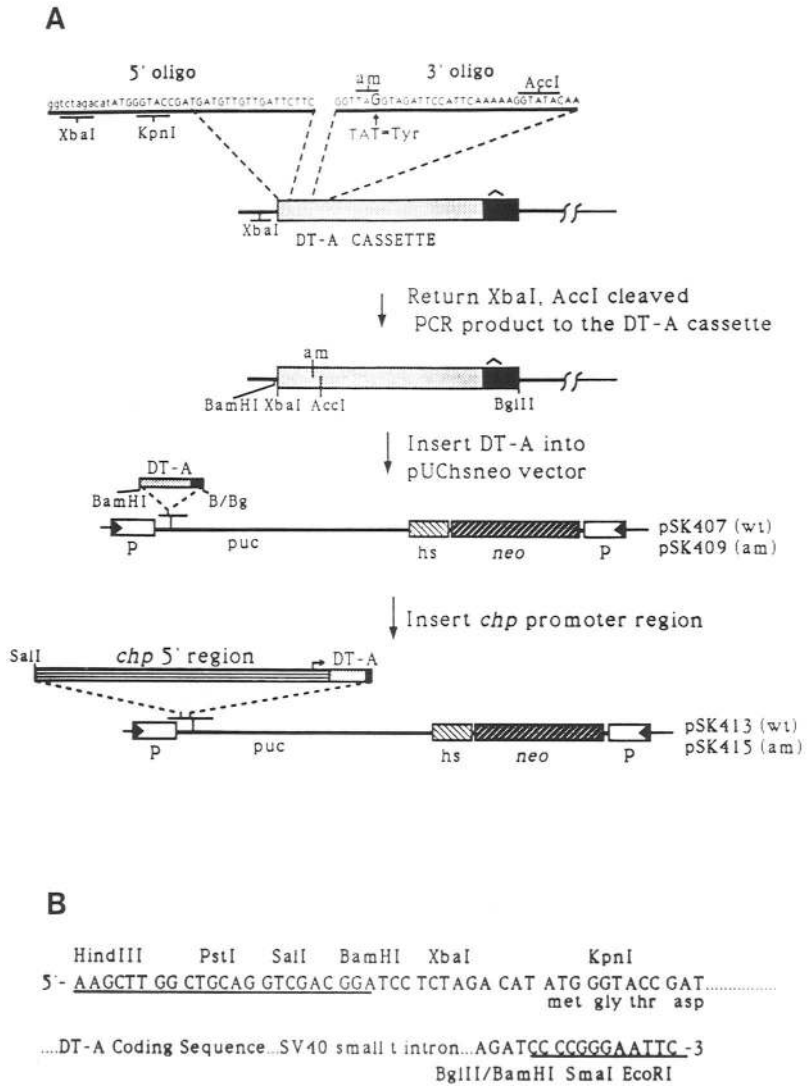


Figure 1. Construction of an amber mutant derivative of the DT-A gene. (*A*) An amber termination codon was introduced into the DT-A chain-coding sequence in a polymerase chain reaction using a mutant oligonucleotide primer. We utilized a 5′ primer that introduces at the 5′ end of the toxin sequence an *Xba*I site, a methionine translation initiation codon, and a *Kpn*I site within the coding sequence. The 3′ primer provides an *Acc*I 3′ end (present in the wild-type DT-A sequence) and an amber (TAG) codon at amino acid residue number 28 (tyrosine, TAT). The reaction was also performed with a wild-type 3′ primer. The products of the reaction performed with the DT-A cassette plasmid as a template were cleaved with *Xba*I and *Acc*I and inserted into the DT-A cassette plasmid. A *Bam*HI–*Bgl*II fragment containing the DT-A gene (light shading) and a 3′ region of SV40 sequence (dark shading) containing the small t intron (^) was inserted into the transformation vector pUChsneo (Steller and Pirrotta 1985) to construct pSK407 (wild-type) and pSK409 (amber). To place the DT-A gene under control of the *chp* promoter, a 4.5-kb *Sal*I–*Xba*I fragment derived from the region immediately upstream of the *chp* open reading frame (Reinke et al. 1988; H. Steller and G.M. Rubin, unpubl.) was inserted into the pUChsneo polylinker upstream of the wild-type and mutant toxin genes, generating pSK413 (wild-type) and pSK415 (amber). This *chp* fragment originates immediately 5′ of the *chp*-coding sequence, ending ~200 bp downstream of the mRNA 5′ end (arrow) and 23 bp upstream of the *chp* translation initiation codon (Reinke et al. 1988). (*B*) Nucleotide sequence of pSK407 and pSK409 at the 5′ polylinker junction of pUChsneo and DT-A, and at the 3′ junction between SV40 sequence and pUChsneo. The modifications introduced by the 5′ PCR primer include the codons for methionine, glycine, and threonine. The fourth codon, aspartic acid, is contained in original DT-A sequence (Maxwell et al. 1986). The changes result in a novel *Kpn*I site within the toxin-coding sequence. The unique restriction sites, *Bam*HI, *Sal*I, and *Xba*I, are indicated.

harboring an amber suppressor tRNA^{Tyr} gene. This suppressor, *P*[*ry*, DtT(Su$^+$)], was constructed previously in vitro and introduced into *Drosophila* by Laski et al. (1989). The structure of compound eyes in freshly eclosed flies harboring the toxin transgene, in the presence and absence of the suppressor tRNA gene, were compared by antidromic illumination (Franceschini 1975) and by light microscopic analysis of plastic sections. When a wild-type head is placed under intense illumination from behind, antidromic light transmission through the eight photoreceptor cells of each ommatidium can be observed externally as seven bright spots in a characteristic trapezoid pattern (Fig. 3C). The central spot is produced by light focused through the central aligned rhabdomeres of photoreceptor cells R7 and R8. The six peripheral spots are contributed by the remaining six photoreceptor cells, R1–6. To examine photoreceptor cells in greater detail, plastic sections of freshly eclosed adults were viewed by light microscopy. A wild-type retinal section is shown in Figure 4A. A normal ommatidial unit contains a core group of eight photoreceptor cells encased by a set of accessory cells consisting of pigment cells, bristle cells, and cone cells (Ready et al. 1976). Only seven photoreceptor cells are detected in a given transverse section, as R7 lies above R8.

In the absence of the amber suppressor gene, some photoreceptor cell defects were detected in the transgenic lines. In three of the lines (3, 11, and 12), mild defects were observed. Approximately 5% of the photoreceptor cells were abnormal when examined by antidromic illumination (arrow in Fig. 3D). The light transmission spots were either missing or highly blurred, aberrations that are not detected in the eyes of either CS or *P*[*ry*, DtT(Su$^+$)]1 flies. Correspondingly, ~5% of the photoreceptor cells were absent from the retinas of *P*[DT-A, *neo*]12 individuals examined in plastic sections. A range

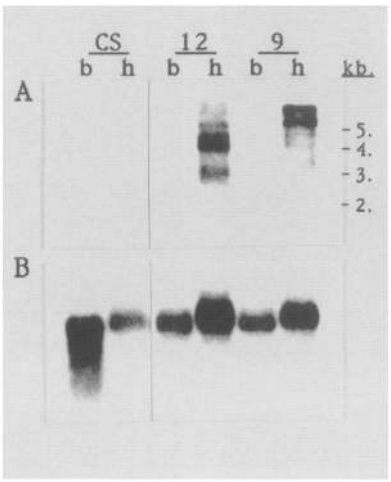


Figure 2. Head-specific expression of toxin mRNA detected by Northern blot analysis. Heads and bodies of freshly eclosed (<12 hr old) adults were separated at the neck and collected for mRNA isolation. Each lane contains polyadenylated RNA isolated from either ~100 heads (h) or 25 bodies (b). (*A*) The filter has been probed with radioactive DT-A cassette plasmid (see Fig. 1). (*B*) To verify the presence of mRNA, the same filter was stripped and hybridized with a probe specific for actin gene transcripts. The lanes correspond to mRNA isolated from CS flies and transgenic lines 9 and 12. The positions of DNA size markers are indicated, and their respective sizes are given in kb.

of more extensive defects were observed in the remaining eight lines. For example, in individuals of *P*[DT-A, *neo*]4 (Fig. 3G), a larger fraction (~10%) of the spots were missing, and those present often appeared weak and diffuse. In several of the lines, as many as 50% of the spots were missing at eclosion. Because diphtheria toxin acts catalytically and is lethal to cells in extremely small amounts (Yamaizumi et al. 1978), rare mistranslation events that lead to the formation of a full-length toxin polypeptide could result in the death of a photoreceptor cell. The frequency with which this occurs presumably reflects the different levels of DT-A transcripts that accumulate in the photoreceptor cells of the various lines.

With the additional presence of the amber suppressor gene, the extent of photoreceptor cell defects was greatly enhanced. For eight transgenic lines (1, 4, 5, 6, 7, 8, 9, and 10), the eyes of offspring appeared opaque and lacked a pupil spot (Fig. 3B). In all of these cases, photoreceptor cells were not detectable by antidromic illumination (e.g., *P*[DT-A, *neo*]4; Fig. 3H) or in plastic sections (*P*[DT-A, *neo*]4 and *P*[DT-A, *neo*]6; Fig. 4C and D, respectively). As can be seen in Figure 4, the photoreceptor cell bodies and their associated rhabdomere specializations were completely absent, while the accessory ommatidial cells remained, forming an empty honeycomb-like structure. In particular, the normal complement of pigment cells and bristle cells were present. In another line (*P*[DT-A, *neo*]12; Fig. 3E and F), most but not all photoreceptor cells were defective when examined by antidromic illumination. For the remaining two lines (*P*[DT-A, *neo*]3 and *P*[DT-A, *neo*]11), ~40–80% of the photoreceptors were absent at eclosion. Consistent with the results of antidromic illumination, in retinal sections of *P*[DT-A, *neo*]3 individuals (Fig. 4B), some photoreceptor cells were normal, others were necrotic, and a considerable fraction was absent.

For nine of the transgenic lines, adults harboring both the toxin and suppressor elements were recovered in the expected Mendelian frequency, indicating that the expression of a functional toxin did not reduce viability. For two lines, *P*[DT-A, *neo*]5 and *P*[DT-A, *neo*]7, suppressor-carrying progeny were significantly under-represented. In both cases, although several suppressor-carrying offspring eclosed late, most died as pupae. Among these offspring, as well as those of the other nine lines, no anatomical defects other than those of photoreceptor cells were observed. In summary, for most (8/11) of the transgenic lines, the presence of the suppressor gene resulted in the complete absence of photoreceptor cells. As anticipated, ablation was confined to the cell population in which the toxin should be expressed.

Photoreceptor cell differentiation is arrested during pupal development

To determine when photoreceptor cell differentiation was arrested in the transgenic strains, we assayed for the expression of photoreceptor cell-specific genes that are normally activated during pupal development. After ~48 hr of pupal development, the antigen recognized by the monoclonal antibody mAb21A6 is first detected associated with photoreceptor cell rhabdomeres (Fujita et al. 1982; Zipursky et al. 1984; Venkatesh et al. 1985). Rh1, the opsin specifically expressed in photoreceptors R1–6, is first synthesized at ~72 hr after puparium formation (Zuker et al. 1985). To assay for cellular protein synthesis after this time point, we used an Rh1–*lacZ* fusion construct, which expresses β-galactosidase under the control of the Rh1 (*ninaE* gene) promoter (Mismer and Rubin 1987, 1989).

Males homozygous for the inserts *P*[DT-A, *neo*]1, *P*[DT-A, *neo*]4, or *P*[DT-A, *neo*]9 were mated to females heterozygous for the amber suppressor insertion *P*[*ry*, DtT(Su$^+$)]1. From this cross, all of the offspring will carry the toxin construct and half will harbor the amber suppressor gene. The heads of 72-hr pupae were stained with both mAb21A6 and the nuclear stain, bis-benzimide (for details, see Materials and methods, below). In the offspring of all three lines, about half of the pupal heads stained with mAb21A6 (e.g., Fig. 5C and G) displayed the normal rhabdomere-localized staining observed in CS pupae (Fig. 5A). However, mAb21A6 staining in the remaining pupae was either very weak or undetectable (Fig. 5E and H, respectively). Moreover, when viewed by bis-benzimide staining, the later class of offspring displayed defects in the position of photoreceptor cell nuclei. In wild-type CS pupal retinas (Fig. 5B) and the offspring that had displayed normal mAb21A6 staining (Fig. 5D and data not shown), the R8 cell nuclei are located in

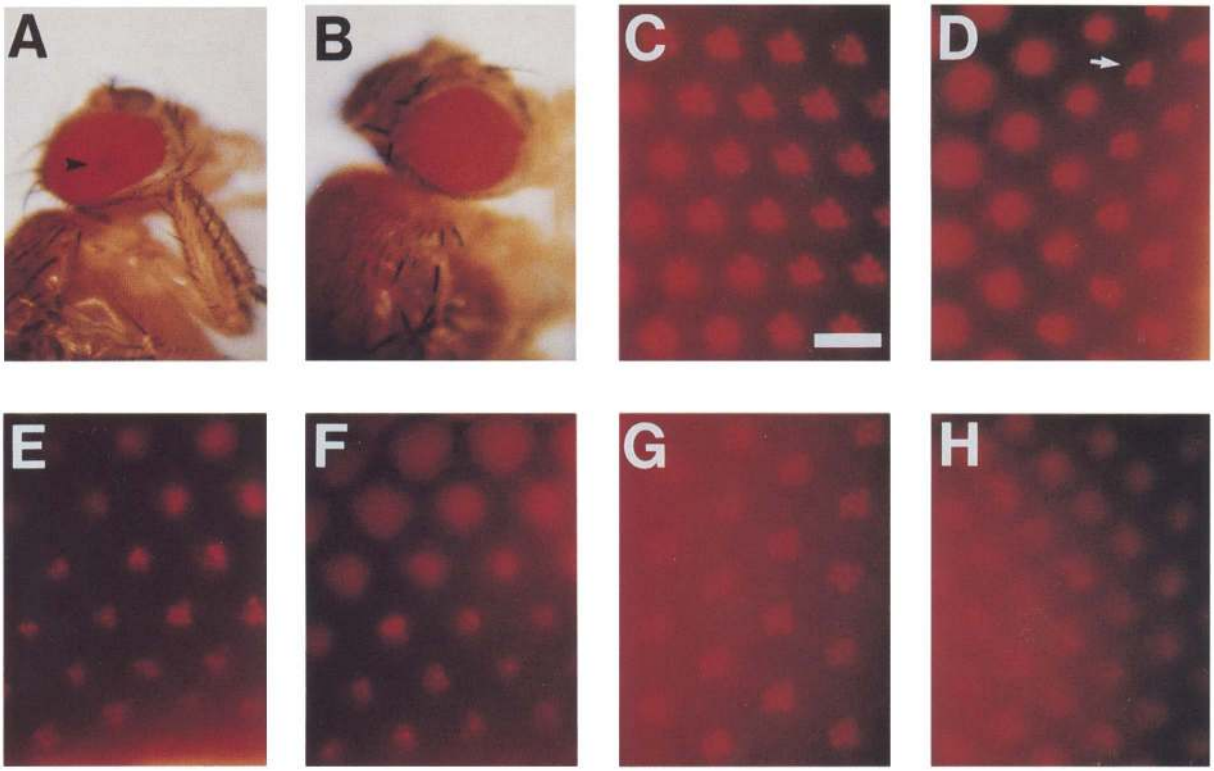


Figure 3. Adult eye phenotype of *P*[DT-A, *neo*] element transformants. A pupil dark spot in the adult retina of CS flies (arrowhead in *A*) is absent in several of the transgenic lines when the amber suppressor tRNA gene has been introduced (*B*). The adult shown in *B* is a ry^+ neo^R offspring from a cross between *P*[DT-A, *neo*]1 and *P*[*ry*, DtT(Su$^+$)]1. Defects in retinula photoreceptor cells were examined by antidromic illumination of adult heads (Franceschini 1975). When an adult head is illuminated from behind and viewed externally by light microscopy, antidromic light conductance by a photoreceptor cell rhabdomere results in a corresponding spot in the dark field. The normal trapezoid array of photoreceptor cell rhabdomeres is seen in *P*[*ry*, DtT(Su$^+$)]1 (*C*). Freshly eclosed adults carrying the *P*[DT-A, *neo*]12 insert appear normal, except for the occasional absence of a single photoreceptor (arrow in *D*). With the presence of both *P*[*ry*, DtT(Su$^+$)]1 and *P*[DT-A, *neo*]12, most photoreceptor cells are defective (*E* and *F*). With the presence of the *P*[DT-A, *neo*]4 insert alone, the spots are weak or occasionally absent (*G*). With the presence of the suppressor in this line, the spots are completely absent (*H*). Bar in *C*, 15 μm (*C–H*).

a well-defined medial row, whereas the R1–R7 photoreceptor and accessory cell nuclei are located apically. However, in the offspring deficient in mAb21A6 staining, a large number of nuclei are located in an aberrant medial position (Fig. 5F and data not shown). We suppose that the pupae of this class harbor the *P*[*ry*, DtT(Su$^+$)]1 insert, whereas the class that displays normal mAb21A6 and bis-benzimide staining do not.

To assess the consequences of toxin expression for the activation of Rh1–*lacZ* expression, females harboring both the *P*[*ry*, DtT(Su$^+$)]1 element and one of the toxin inserts, *P*[DT-A, *neo*]1, *P*[DT-A, *neo*]4, or *P*[DT-A, *neo*]9, were crossed to males of either of two strains homozygous for Rh1–*lacZ* element insertions (Mismer and Rubin 1987). Because the offspring are selected for neo^R, all will harbor both the toxin element and the Rh1–*lacZ* gene and half will harbor the amber suppressor gene. These offspring were assayed for β-galactosidase activity in sections from 96-hr-old pupae. For the crosses involving inserts *P*[DT-A, *neo*]1 and *P*[DT-A, *neo*]4, two classes of offspring were observed. In one class, the staining of retinal sections appeared indistinguishable from the offspring recovered from crossing either of the Rh1–*lacZ* strains to ry^{506} females (cf. Fig. 5I with K). These offspring thus appear to be fully capable of protein synthesis at the 72-hr time point. The second class of offspring were either completely or almost completely devoid of staining (Fig. 5J and L). In the latter cases, staining could be detected in what appeared to be single photoreceptor cells (arrowheads, Fig. 5L). We suppose that the presence or absence of β-galactosidase activity in these offspring corresponds to the absence or presence, respectively, of the amber suppressor gene. With one line (*P*[DT-A, *neo*]9), we observed offspring that entirely lacked staining and others that stained more weakly than seen with the Rh1–*lacZ* element alone (data not shown). Perhaps with this insertion, there is sufficient toxin expression despite the suppressor's absence to arrest protein synthesis shortly after the onset of Rh1–*lacZ* expression.

In summary, we have found that expression of the DT-A gene under the control of the *chp* promoter inhibits the synthesis of two photoreceptor cell-specific differen-

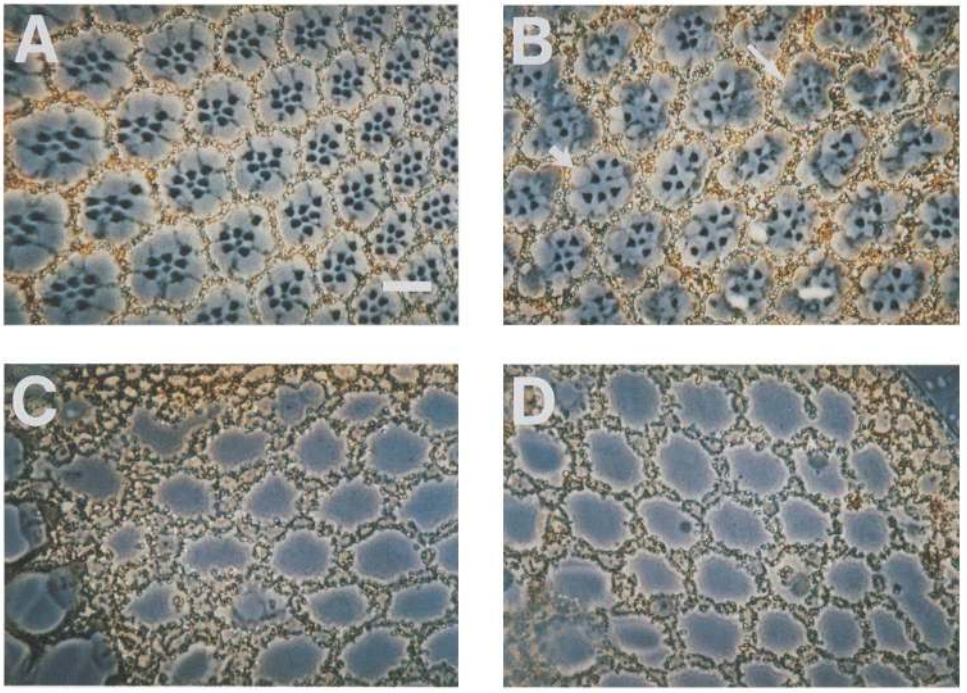


Figure 4. Light microscopic analysis of adult eyes in plastic sections. The heads of freshly eclosed adults were embedded in plastic and sectioned. Semithin sections (1 μm) were viewed by phase-contrast microscopy. A transverse section of a CS (wild-type) retina is shown in *A*. In a given plane of section, seven darkly stained rhabdomeres and their surrounding photoreceptor cell bodies are visible within an ommatidium. Each photoreceptor cell ensemble is encased by a shared set of pigment cells, which display characteristic pigment granules. In the presence of the suppressor gene, *P*[DT-A, *neo*]3 displays incomplete ablation (*B*), consistent with the results of antidromic illumination (data not shown). Some ommatidia appear to contain a normal complement of photoreceptor cells (short arrow), whereas others display necrotic cells (long arrow). In many ommatidia, only a few cells appear necrotic. With the presence of the suppressor gene, the eyes of *P*[DT-A, *neo*]4 (*C*) and *P*[DT-A, *neo*]6 (*D*) flies are completely devoid of photoreceptor cells. The retinal accessory cells remain, forming an empty honeycomb-like structure. The absence of photoreceptor cells appears to allow the pigment cells to expand in width. Bar in *A*, 10 μm (*A–D*).

tiation markers, mAb21A6 and rhodopsin. This suggests that DT-A expression arrests photoreceptor cell development by 48 hr of pupal development.

Ablation of the larval photoreceptor nerve (Bolwig's nerve)

Because *chp* is expressed in all photoreceptor cells, including the larval photoreceptor cells (Zipursky et al. 1984), the *chp* promoter should confer toxin expression in these cells as well. To detect the ablation of the larval photoreceptor nerve in the transgenic lines, we examined third-instar larvae by immunocytochemical staining with mAb24B10, which binds to chaoptin, the *chp* gene product. In a normal CS larva (Fig. 6A) mAb24B10 labels the larval photoreceptor nerve and the cell bodies and axons of the retinula photoreceptor cells in the developing eye–imaginal disc and optic lobe. The onset of *chp* expression in the retinula photoreceptors occurs several hours after passage of the morphogenetic furrow. Chaoptin is detected in photoreceptor cell axons soon after they enter the optic lobe primordium.

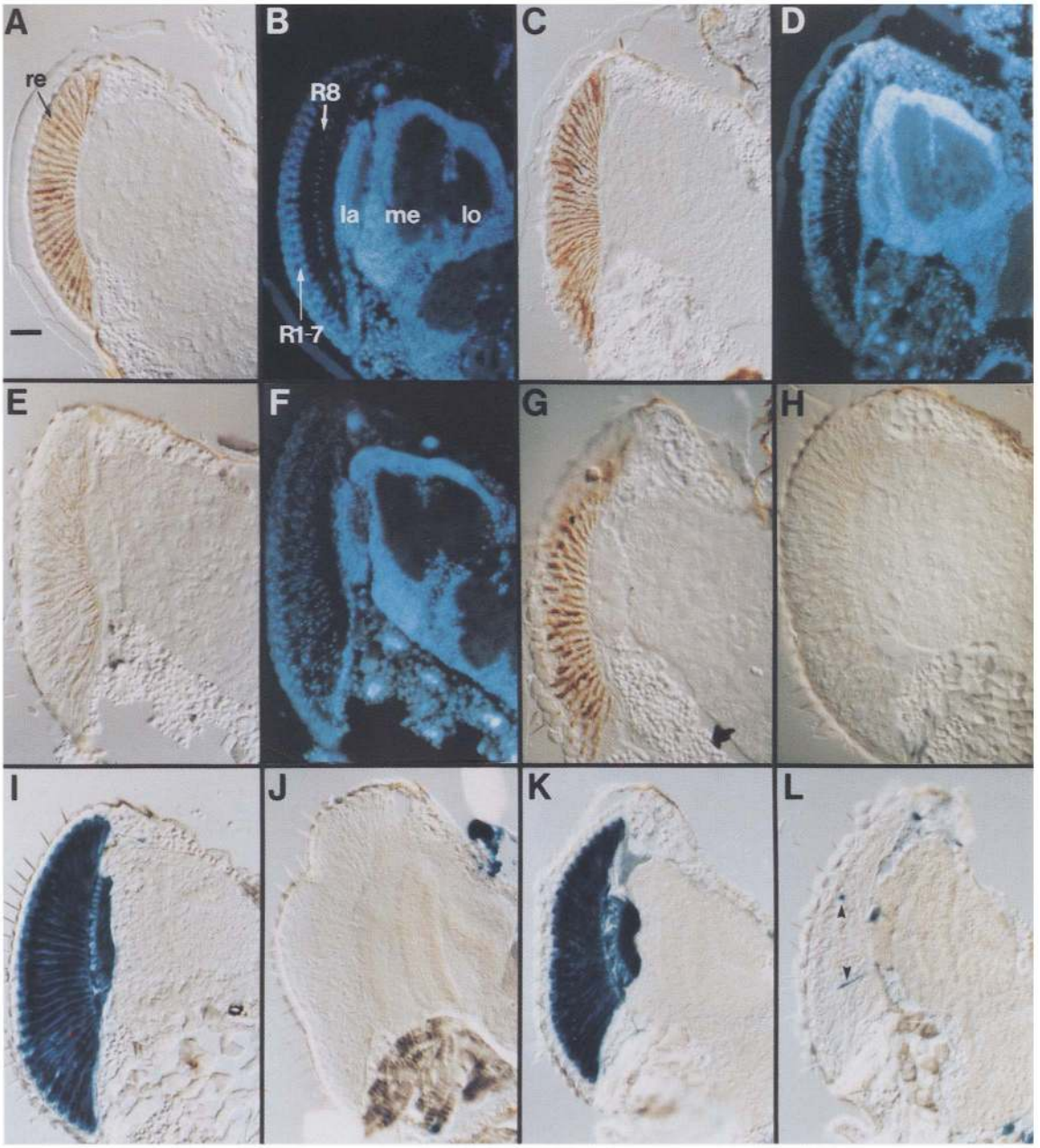
In five of the six lines examined, the presence of the amber suppressor gene resulted in defects in the larval photoreceptor nerve. In suppressor-carrying offspring of *P*[DT-A, *neo*]9 (Fig. 6D), the larval photoreceptor nerve was greatly reduced in diameter and appeared broken at multiple sites. In the progeny of *P*[DT-A, *neo*]5 and *P*[DT-A, *neo*]3 (Fig. 6B and C, respectively), the larval photoreceptor nerve was not detectable. The occasional presence of a faint isolated remnant of the nerve, or residual staining at the site of its synapse in the optic lobe, suggests that the nerve was present at an earlier stage. Progeny of *P*[DT-A, *neo*]4 and *P*[DT-A, *neo*]7 exhibited moderately reduced staining of the larval photoreceptor nerve (data not shown). In all of the lines examined, *chp* expression appeared normal in retinula photoreceptor cells (Fig. 6B, C–D). The presence of the *chp* product in these cells is consistent with the notion that protein synthesis does not cease immediately after the onset of toxin expression.

Effects of photoreceptor cell ablation on the structure of adult optic ganglia

Studies with mutants partially or completely lacking photoreceptor cells have revealed that innervation by the larval and retinula photoreceptor cells is a critical re-

quirement for the development of the adult optic ganglia (Power 1943; Meinertzhagen 1973; Meyerowitz and Kankel 1978; Nassel and Sivasubramanian 1983; Fischbach 1989). When both sets of neurons do not innervate the brain, as occurs in the mutant *disconnected* (*disco*) (Steller et al. 1987), the optic lobe is virtually absent in the adult; muscle and hemolymph replace optic lobe neuronal tissue. When the optic lobe lacks only innervation by the retinular photoreceptor cells, as occurs in eyeless individuals of the *sine oculis* mutant, an optic lobe of reduced volume results (Fischbach 1983). The first optic ganglion, the lamina, is entirely absent, and the medulla and lobula complex are reduced in size (Fig. 8B, below). It has recently been shown that photoreceptor cell innervation is required for the initial steps of lamina neurogenesis that occur in the late third-instar and early pupal stages (Selleck and Steller 1991). If photoreceptor cells only play an early and transient role, their ablation by midpupation might not prevent the development of normal optic ganglia.

To further define the role of photoreceptor cell innervation in optic lobe development, we examined the optic ganglia of adults in which photoreceptor cells had been ablated. To visualize photoreceptor cell axonal projec-



(**Figure 5.** *See facing page for legend.*)

tions into the optic ganglia, frozen sections from adult heads were stained with the anti-chaoptin antibody, mAb24B10, which binds specifically to retinular photoreceptor cell bodies and axons (Zipursky et al. 1984; Fig. 7A) . To visualize the optic ganglia, the sections were stained with anti-horseradish peroxidase (HRP) antibodies, which bind to all neurons, including photoreceptor cells (Jan and Jan 1982; Fig. 8A and E) . The lamina was examined in finer detail by light microscopy of plastic sections (Fig. 9) .

When the suppressor gene was present, eight lines were found to lack both mAb24B10 and anti-HRP staining in the retina, except for the residual staining of apparent cellular debris adjacent to the basement membrane (Fig. 7B–D; Fig. 8C–G). These eight lines had also shown complete ablation when examined by antidromic illumination and light microscopy of plastic sections. Surprisingly, in these lines, the photoreceptor axonal fibers could still be observed by mAb24B10 staining, though the intensity of staining was often moderately diminished (cf. Fig. 7, B–D, with the wild-type section, A). Either the photoreceptor axons have remained intact in the absence of their cell bodies or the stained material represents only axonal debris. As can be seen in Figure 7, B–D, the axonal projections into the optic lobe appear normal. The suggestion that these individuals possess normal optic ganglia is further supported by the results of anti-HRP antibody staining (Fig. 8). The lamina, the medulla, and the lobula complex are of approximately normal size and organization (cf. Fig. 8E with F). The presence of a normally organized lamina is clearly revealed by light microscopic examination of plastic sections (Fig. 9). In particular, it is possible to discern the presence of lamina interneurons organized into optic cartridges, the repeated units in which the axons of R1–6 synapse with lamina interneurons (see, e.g., Trujillo-Cenóz and Melamed 1966; for review, see Strausfeld 1976).

With either antibody, the three lines that had displayed incomplete retinula photoreceptor cell ablation (*P*[DT-A, *neo*]3, *P*[DT-A, *neo*]11, and *P*[DT-A, *neo*]12) exhibited an approximately normal staining pattern in both the retina and optic lobe, although frequent breaks in the retinal tissue indicate an unusual mechanical weakness (Fig. 7E and data not shown). It is worth noting that normal optic ganglia were observed in the two lines (*P*[DT-A, *neo*]3 and *P*[DT-A, *neo*]5) that appear to lack the larval photoreceptor nerve in the third instar. Thus, the formation and maintenance of normal optic ganglia does not appear to require the continuous presence of intact larval or retinula photoreceptor cells.

Discussion

We have used toxin-mediated ablation to study some aspects of visual system development in *Drosophila*. In our attempt to recover germ-line transformants with a diphtheria toxin construct, we found it necessary to prevent transient toxin gene expression from microinjected DNA. A plasmid bearing a functional DT-A gene killed nearly all microinjected embryos. Such lethality would not be expected if, in microinjected embryos, toxin expression was properly restricted to photoreceptor cells by the *chp* promoter. Unsuccessful attempts to recover toxin gene transformants have also been made in other laboratories [Tze-Bin Chou (Harvard Medical School) pers. comm.; C. Zuker (University of California, San Diego), pers. comm.]. Because extremely small amounts of the toxin can be lethal, at least in mammalian cells (Yamaizumi et al. 1978), even low levels of aberrant transient expression could prove lethal to an embryo. Indeed, although the localization of transcripts synthesized from DNA microinjected into *Drosophila* embryos generally reflects the tissue specificity of the promoter used, a small amount of aberrant expression is detected (Steller and Pirrotta 1984). In contrast, there has been substan-

Figure 5. Arrest of photoreceptor cell development during pupation. To determine whether DT-A gene expression prevents the expression of the photoreceptor cell-specific 21A6 antigen, males homozygous for the inserts *P*[DT-A, *neo*]1, *P*[DT-A, *neo*]4, or *P*[DT-A, *neo*]9 were crossed to females harboring the *P*[*ry*, DtT(Su$^+$)]1 insert. The heads of 72-hr-old pupae were sectioned in a cryostat and stained with the mouse monoclonal antibody mAb21A6 and the nuclear-specific stain bis-benzimide (Hoechst H 33258). (*A*) mAb21A6 staining of a CS retina (re). mAb21A6 staining is localized to rhabdomeres (Zipursky et al. 1984) (*B*) Bis-benzimide nuclear staining of the CS retinal section shown in *A*. The medial row of R8 nuclei, the apical row of R1–7 and accessory cell nuclei, and the lamina (la), medulla (me), and lobula complex (lo) are indicated. (*C*) Normal offspring from the cross with *P*[DT-A, *neo*]1 stained with mAb21A6. (*D*) Bis-benzimide staining of the retinal section shown in *C*. Note the normal location of retinal nuclei. (*E*) Abnormal offspring from the cross with *P*[DT-A, *neo*]1, showing severely reduced staining with mAb21A6. (*F*) Bis-benzimide staining of the section shown in *E*. In the retina, nuclei are located in aberrant medial positions. However, the optic ganglia appear normal. (*G*) Normal offspring from the cross with *P*[DT-A, *neo*]4 stained with mAb21A6. (*H*) Offspring from the cross with *P*[DT-A, *neo*]4, which virtually lacks staining with mAb21A6. To determine whether the toxin affects the normal expression of an Rh1–*lacZ* fusion gene, females harboring both the toxin and amber suppressor elements were crossed to males homozygous for either of two insertions of the *P*[*ry*; Rh1(−252/+67)–*lacZ* : omSMB] element (Mismer and Rubin 1989). Neo^R offspring were sectioned in a cryostat after 96 hr of pupation. Sections were stained for β-galactosidase activity with the chromogenic indicator 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Bachem). (*I*) Section through the retina of an offspring from a cross of *P*[*ry*; Rh1(−252/+67)–*lacZ* : omSMB] males and *ry*^{506} females. These offspring (30 of 30 tested) exhibit normal Rh1–*lacZ* expression in the retina and the R1–6 axons in the lamina. (*J*) Offspring harboring *P*[DT-A, *neo*]1, which lacks Rh1–*lacZ* expression in the retina and lamina. (*K*) Offspring harboring *P*[DT-A, *neo*]4, which displays normal Rh1–*lacZ* expression in the retina and lamina. (*L*) Offspring harboring *P*[DT-A, *neo*]4, which lacks Rh1–*lacZ* expression, except in isolated positions (arrowheads). These sites may correspond to photoreceptor cells that have escaped ablation. Horizontal sections are shown (*A*—*L*), with anterior at *bottom* and lateral toward the *left*. Bar in *A*, 30 μm (*A*–*L*).

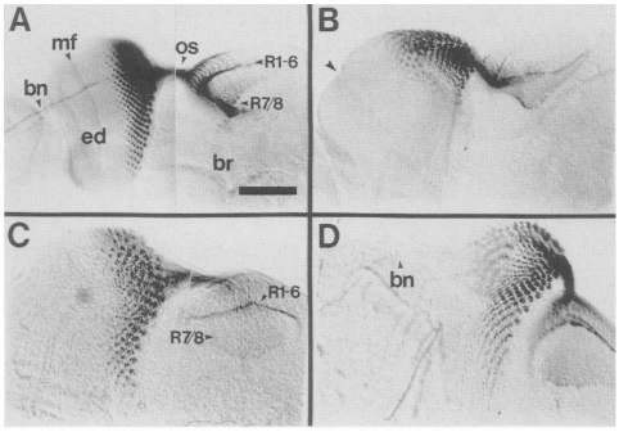


Figure 6. Immunocytochemical visualization of the larval and retinula photoreceptor cells in third-instar larvae. The *P*[DT-A, *neo*] transgenic lines were crossed to the amber suppressor strain *P*[*ry*, DtT(Su$^+$)]1. The eye–antennal discs and brains of neo^R third-instar larvae were stained with mAb24B10. A CS larval whole mount is viewed in a pastiche of two focal planes in *A*. The larval photoreceptor nerve (bn) is seen running through the eye–antennal disc (ed), through the optic stalk (os), and into the brain (br). Its terminus in the brain is also visible. Retinula photoreceptor cells, which express chaoptin (the 24B10 antigen) several hours after the passage of the eye morphogenetic furrow (mf), stain strongly in the posterior third of the eye disc. Their axons pass through the optic stalk and terminate in the brain (R1–6 in the lamina primordium, and R7/8 in the medulla primordium). From the cross with *P*[DT-A, *neo*]5 (*B*) and *P*[DT-A, *neo*]3 (*C*), the larval photoreceptor nerve is absent in about half of the neo^R progeny. The morphogenetic furrow is indicated by an arrowhead in *A* and *B*. In about half of the neo^R progeny of *P*[DT-A, *neo*]9 (*D*), the larval photoreceptor nerve is highly thinned and broken at multiple positions. Anterior is at *left*; lateral is at *top*. Bar in *A*, 75 μm (*A–D*).

tially less difficulty in recovering transgenic mice bearing toxin genes (Palmiter et al. 1987; Behringer et al. 1988; but see also Landel et al. 1988).

To overcome this difficulty, we introduced an amber mutation into the DT-A-coding sequence, placing the toxin's synthesis under conditional control. With the mutant DT-A gene, survival following microinjection was 10-fold greater than with the wild-type gene, thus permitting the recovery of a germ-line transformant. Although the frequency of transformants in this case is low (one among 83 G_0 adults), a high frequency of transformants was recovered with a construct containing the mutant DT-A gene under the control of a different promoter (Tze-Bin Chou, pers. comm.).

Conditional ablation of photoreceptor cells

The effects of the toxin transgene were found to depend on the presence of an amber suppressor tRNA gene. In the absence of the amber suppressor, significant levels of toxin gene transcripts accumulate in the adult head. Apparently, photoreceptor cells can tolerate these mutant transcripts for at least several days. The mutant transcripts did not prevent the expression of two photoreceptor cell-specific markers, the 21A6 antigen and an Rh1–*lacZ* fusion gene, normally activated during pupal development. However, some photoreceptor cell defects were observed in adults. These effects likely reflect the occasional synthesis of a full-length mistranslated toxin protein. Because a single toxin protein molecule is sufficient to kill a mammalian cell (Yamaizumi et al. 1978), such products could be rare and yet bring about the death of a photoreceptor cell. When the amber suppressor gene was present, the expression of both the 21A6 antigen and the Rh1–*lacZ* gene was almost totally eliminated. Thus, in the presence of the suppressor, it appears that photore-

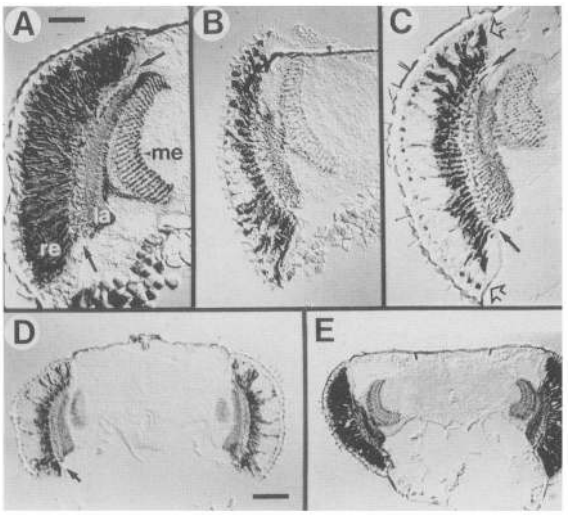


Figure 7. Immunocytochemical visualization of retinula photoreceptor cells in adult head sections. The heads of freshly eclosed (<12 hr old) adults were sectioned and stained with either mAb24B10 or FITC-conjugated goat anti-HRP antibodies (Fig. 8). (*A*) Frontal section showing the retina and optic ganglia of a CS fly. mAb24B10 stains photoreceptor cell bodies in the retina (re), and the R1–6 and R7/8 photoreceptor axons in the lamina (la) and medulla (me), respectively. (*B*) Section of the eye and optic ganglia of an adult harboring *P*[DT-A, *neo*]4 and *P*[*ry*, DtT(Su$^+$)]1. Lateral areas of the retina are devoid of staining. The medial region, adjacent to the basement membrane, shows staining of apparent cellular debris. In the lamina and medulla, the photoreceptor axons stain with reduced intensity but project in an apparently normal pattern. (*C*) Section of the eye and optic ganglia of an adult harboring *P*[DT-A, *neo*]6 and *P*[*ry*, DtT(Su$^+$)]1. The photoreceptor axon pattern in the medulla differs from that shown in *A* owing to the plane of section. (*D*) Lower magnification photograph of a section from the head of an adult harboring *P*[DT-A, *neo*]6 and *P*[*ry*, DtT(Su$^+$)]1. (*E*) Section from the head of an adult harboring *P*[DT-A, *neo*]3 and *P*[*ry*, DtT(Su$^+$)]1. Consistent with the examination of this line by antidromic illumination and by phase contrast microscopy of plastic sections, the retinal staining is almost normal, except for the presence of breaks in the tissue. The lamina and medulla appear normal. Solid arrows (*A*, *C*, *D*) indicate the position of the basement membrane. Open arrows (*C*) indicate the distal area of the retina that is devoid of mAb24B10 staining. (*A–C*) Dorsal is at *top*; lateral is at *left*. Bar in *A*, 50 μm (*A–C*); bar in *D*, 100 μm (*D–E*).

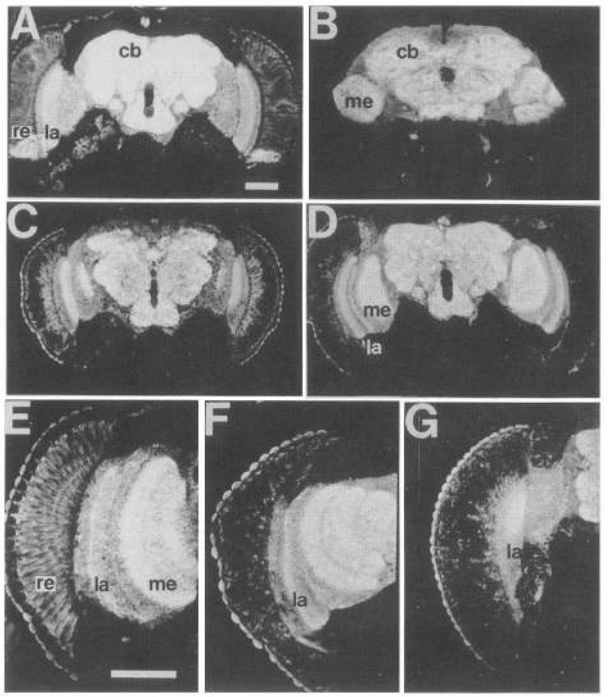


Figure 8. Immunocytochemical visualization of optic ganglia in adult head sections. Frontal sections of adult heads (see Fig. 7) were stained with FITC-conjugated goat anti-HRP antibody. (*A*) A CS section stains intensely in the central brain (cb) and the lamina (la) and more weakly in the retina (re). (*B*) In an eyeless *sine oculis* adult, the retina and lamina are absent and the medulla is reduced in size relative to the wild type (cf. *E*; see also Fischbach 1983). (*C*) Section from an adult harboring *P*[DT-A, *neo*]4 and *P*[*ry*, DtT(Su$^+$)]1. Note the absence of retinal staining except in the medial region adjacent to the basement membrane, while the lamina stains normally. (*D*) Section from an adult harboring *P*[DT-A, *neo*]6 and *P*[*ry*, DtT(Su$^+$)]1. As in *C*, retinal staining is almost absent, while the lamina (la) and medulla (me) stain normally (cf. *E*). (*E*) Section from a CS head at higher magnification than in *A*. (*F*) Section from an adult harboring *P*[DT-A, *neo*]6 and *P*[*ry*, DtT(Su$^+$)]1 at higher magnification than in *D*. (*G*) Section from an adult harboring *P*[DT-A, *neo*]8 and *P*[*ry*, DtT(Su$^+$)]1. Note the absence of retinal staining and the presence of the lamina. This section is more anterior than that shown in *E* and does not include the medulla. (*A–D*) Dorsal is at *top*; (*E—G*) dorsal is at *top*; lateral is at *left*. Bar in *A*, 100 μm (*A–D*); bar in *E*, 100 μm (*E–G*).

ceptor cell development ceases by the end of the second pupal day. In most of the transgenic lines, photoreceptor cell bodies were absent from the retinas of freshly eclosed adults. Thus, despite the fact that the *P*[*ry*, DtT(Su$^+$)] transgene provides <1% amber suppression (Laski et al. 1989), the resulting level of toxin activity is clearly sufficient to achieve cellular ablation in *Drosophila*, at least in a case where the toxin mRNA is abundantly expressed.

To ablate specific cells, the expression of the toxin gene must be precisely restricted to the targeted cell population, and once synthesized, the toxin must act cell-autonomously. Consistent with the well-documented expression pattern of the *chp* gene (Zipursky et al. 1984), photoreceptor cell ablation was the only anatomical defect observed in nine transgenic lines harboring the toxin element at different genomic locations. In two other lines, we observed reduced viability at the pupal stage, presumably as a result of ectopic DT-A expression in cells essential for the viability of the organism. This position dependence is consistent with the general observation that, when using essentially complete promoter sequences, chromosomal context often influences the level of transgene expression but usually not its tissue specificity (Spradling and Rubin 1983; Bourouis and Richards 1985; Wakimoto et al. 1986). The toxin was expected to act cell autonomously because the DT-A gene does not encode the second subunit of native diphtheria toxin that facilitates transit across cellular mem-

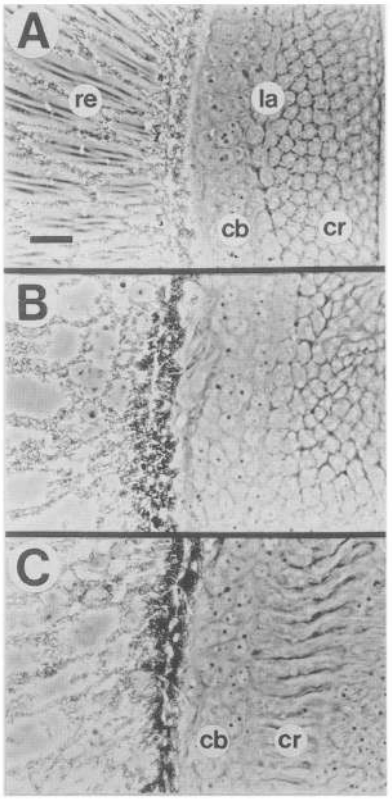


Figure 9. Lamina structure of adults with ablated photoreceptor cells. Semithin frontal sections from the plastic-embedded heads of freshly eclosed adults were viewed by phase-contrast microscopy. (*A*) Section from a CS fly showing normal ommatidia in the retina (re), and lamina neuron cell bodies (cb) and cartridges (cr) in the lamina ganglion. In this view, the cartridges are seen in transverse section. (*B*) Section through the retina and lamina of an adult harboring the *P*[DT-A, *neo*]4 and *P*[*ry*, DtT(Su$^+$)]1 elements. Photoreceptor cell bodies are absent from the retina, but the lamina cell bodies and cartridges are present. (*C*) Section from the same eye and brain as shown in *B* showing lamina cartridges in longitudinal section. Lamina neurons can be seen to send axons toward the deeper optic ganglia. Bar in *A*, 10 μm (*A–C*).

branes (Uchida et al. 1973; Pappenheimer 1977). Our observations are consistent with this expectation. Despite DT-A gene expression in retinula photoreceptor cells, the neighboring pigment and bristle cells, many of which closely contact photoreceptor cells in the pupal and adult eye (Ready et al. 1976; Tomlinson and Ready 1987; Cagan and Ready 1989), remained intact. While the photoreceptor cells ceased development by the end of the second pupal day, pigment and bristle cell development appeared to proceed unaffected to the adult stage. Thus, although the toxin may be released by cell lysis to the extracellular milieu, it does not appear to gain entry into surrounding cells.

Photoreceptor cell innervation and the development of the optic lobe

The larval photoreceptor nerve, part of the larval visual system, is thought to serve a pioneer role in the establishment of neuronal connectivity between the adult eye and brain (Meinertzhagen 1973, 1974; Steller et al. 1987; Fischbach et al. 1989). In the embryo, the larval photoreceptor nerve forms stable synaptic connections with target cells in the optic lobe anlagen. It is sheathed in an epithelial invagination, the optic stalk, that connects each eye–imaginal disc with the ipsilateral larval brain hemisphere. As retinula photoreceptor cells differentiate during the third instar, a fascicle derived from each ommatidial cluster extends down the optic stalk alongside the larval photoreceptor nerve and enters the optic lobe anlage. An essential role for the larval photoreceptor nerve in this process is suggested by the analysis of the mutation *disconnected* (Steller et al. 1987). In *disco* embryos, the larval photoreceptor nerve fails to establish stable synaptic connections with its target cells and the optic stalk is absent. Consequently, retinula photoreceptor axons, unable to enter the brain, remain in the eye–imaginal disc.

The mechanism that guides retinula photoreceptor axons into the optic stalk and to their appropriate targets in the brain remains unknown. The larval photoreceptor nerve might play a key role in this process by guiding the retinula cell axons to their optic lobe anlage targets, as has been suggested by Meinertzhagen (1973, 1974). On the other hand, the retinula axons may rely on other environmental guidance cues, for example, components of the basal lamina surrounding the eye disc and optic stalk (see Sanes et al. 1978; Dodd and Jessell 1988; Eisen 1988). In two transgenic lines in which the larval photoreceptor nerve appeared to be absent during the third instar, retinula cell axons entered the optic anlage and achieved an apparently normal projection pattern. In adults, the remnants of retinula cell axons were connected to apparently normal optic ganglia. Although these observations suggest that the larval photoreceptor nerve is dispensable for retinula axon pathfinding, we cannot rule out the possibility that these axons follow remnants of the nerve that remain and are not detected by the antibody staining.

In mutants lacking retinula photoreceptor cells, the first optic ganglion, the lamina, is absent from the adult brain, and the medulla and lobula optic neuropils are hypotrophic (Power 1943; Meinertzhagen 1973; Fischbach 1983). These defects arise during late larval and pupal development in association with the failure of photoreceptor cell axons to arrive in the anlage. In the absence of innervating photoreceptor cell axons, lamina neurogenesis does not occur (Selleck and Steller 1991), and the anlage undergoes extensive cell death (Fischbach and Technau 1984). The developmental mechanisms underlying this dependence remain unclear. In particular, it is not known whether normal development requires transient or continuous photoreceptor cell innervation. If this role is transient, it would be useful to determine the specific time at which the eye–brain interaction occurs.

Our analysis indicates that blocking retinula photoreceptor cell development after their axons arrive in the brain does not prevent the formation of apparently normal optic ganglia. Despite the arrest of photoreceptor cell development by the end of the second pupal day, the adult lamina is present and indistinguishable from the wild type at the resolution of light microscopic analysis of plastic sections. In particular, the lamina is organized in the repetitive array of cartridges within which photoreceptor cell axons synapse with lamina interneurons (for details of lamina structure, see Trujillo-Cenóz and Melamed 1966; Strausfeld 1976). This is in striking contrast to the complete absence of these structures in eyeless mutants. Moreover, these flies have medulla and lobula complexes of apparently normal volume and organization. Presumably, more severe optic lobe defects, comparable to those observed in mutants lacking retinal innervation, require the ablation of photoreceptor cells earlier in their development, perhaps before their axons enter the brain.

The use of conditional ablation in Drosophila

Placing toxin expression under conditional control will offer important advantages in addition to permitting the recovery of germ-line transformants. For example, because the toxin mRNA can be detected under the nonpermissive condition, one could verify the anticipated developmental expression pattern of a particular toxin gene fusion by tissue in situ RNA hybridization analysis. Additionally, it may be possible to recover and maintain transgenic lines in which the toxin can be expressed in tissues that are essential for the survival of the organism. This would extend the use of toxin-mediated ablation to include the analysis of cell–cell interactions in the development of tissues which, unlike the eye, are not dispensable. Whether the leaky toxin expression that occurs without the amber suppressor will prevent the survival of such lines is not clear. Several strategies to reduce the background level of toxin expression are currently being explored. By using the enhancer-trap methodology of O'Kane and Gehring (1987; see also Bellen et al. 1989) to identify promoter elements that confer a desired developmentally specific pattern of expression, it

should be possible to ablate a cell population of choice. Given this possibility, we expect that conditional toxin-mediated ablation methods will find wide application in the study of development and behavior in *Drosophila*.

Materials and methods

Drosophila *culture*

All fly strains were grown on standard cornmeal medium (Cline 1978) at 18°C or 25°C. Selection for neomycin resistance (neo^R) was performed by placing adult flies on food containing 0.7 mg/ml of G418 (Geneticin, GIBCO Laboratories; for further details, see Steller and Pirrotta 1985.)

Recombinant DNA methods

An amber mutation was introduced into the DT-A gene in a polymerase chain reaction using a mutant oligonucleotide primer (see legend to Fig. 1). The reaction was carried out under standard conditions (Cetus) using *Hind*III-linearized DT-A cassette as the template. The resulting modifications were confirmed by nucleotide sequence analysis. The recombinant DNA methods employed in subsequent steps in the construction (Fig. 1) were performed essentially as described in Sambrook et al. (1989).

Germ-line transformation

Embryos of the strain yw^{67c23} were injected with the toxin plasmids, along with the helper plasmid, pπ25.7wc (Karess and Rubin 1984). With two independent DNA preparations of the wild-type pSK413 plasmid, a total of 35 of 624 injected embryos survived to the first-instar larval stage. Seventeen adults were subsequently recovered. With the mutant toxin plasmid, pSK415, 168 first-instar larvae were recovered from 337 injected embryos. Eighty-three adults subsequently eclosed. Selection was made for germ-line transformants by crossing the adults to ry^{506} animals on G418-containing food (Steller and Pirrotta 1985). No neo^R offspring were recovered from the 17 adults surviving injection with the wild-type construct. One transformant line, denoted *P*[DT-A, *neo*]12, was recovered from the 83 adults surviving injection with the mutant toxin construct. To recover toxin element insertions at other chromosomal locations, transposition was induced by crossing *P*[DT-A, *neo*]12 to a strain harboring *P*[*ry*, Δ2-3]99B (Laski et al. 1986; Robertson et al. 1988), which provides a constitutive source of *P* transposase. Ten independent transposant lines were recovered: *P*[DT-A, *neo*]1, *P*[DT-A, *neo*]3, *P*[DT-A, *neo*]4, *P*[DT-A, *neo*]5, *P*[DT-A, *neo*]6, *P*[DT-A, *neo*]7, *P*[DT-A, *neo*]8, *P*[DT-A, *neo*]9, *P*[DT-A, *neo*]10, and *P*[DT-A, *neo*]11. The various *P*[DT-A, *neo*] transgenic lines were crossed into a *ry* background, so that the presence of the amber suppressor element *P*[*ry*, DtT(Su^+)]1 could be detected; ry^+ neo^R offspring harbor both the toxin transgene and the suppressor tRNA gene.

RNA isolation and analysis

Heads and bodies of freshly eclosed (<12 hr old) adults were separated at the neck and frozen on dry ice. Polyadenylated RNA was isolated from homogenized tissue using the Fast Track System (Invitrogen). Northern analysis was performed by formaldehyde gel electrophoresis of RNA and transfer to a nitrocellulose filter membrane, essentially as described by Sambrook et al. (1989).

Plastic sections for light microscopy

Adult heads were cut at the neck, and their proboscides were removed in a fixative solution containing 1% glutaraldehyde, 2% formaldehyde, and 0.1 M phosphate (pH 7.4). Fixation continued for 1 hr at room temperature. Heads were postfixed in 2% osmium tetroxide and 0.1 M phosphate (pH 7.4) for 30 min and embedded in Spurr's medium (Spurr 1969). Semithin (1 μm) sections were examined by phase-contrast microscopy.

FITC and HRP immunocytochemistry

For mAb24B10 antibody staining, late third-instar (climbing) larvae were dissected in 0.1 M phosphate buffer (pH 7.4) and fixed for 10 min in the dark in 0.2% benzoquinone, 0.1 M phosphate (pH 7.4). Subsequent steps were carried out as described by Steller et al. (1987), with the exception that balanced saline solution (Ashburner 1989) was substituted for PBS. The secondary antibody, HRP-conjugated goat anti-mouse IgG (Bio-Rad), was used at a dilution of 1 : 100.

Cryostat sections of adult and pupal heads were prepared for immunocytochemistry as described by Selleck and Steller (1991). Antibody staining was performed as described by Steller et al. (1987). mAb24B10 hybridoma supernatant, mAb21A6 ascites fluid, and FITC-conjugated goat anti-HRP (Cappel) were used at dilutions of 1 : 1, 1 : 200, and 1 : 100, respectively. For FITC immunofluorescence, sections were mounted in PBS, 70% glycerol, and 0.1% phenylenediamine (Johnson and Araujo 1981) and viewed by epifluorescence. mAb24B10 and mAb21A6 binding was visualized by incubation with HRP-conjugated goat anti-mouse IgG (Bio-Rad) and peroxidase staining as described by Steller et al. (1987). To visualize cell nuclei, sections were immersed for 5 min in 1 μg/ml of bis-benzimide (Hoescht H 33258) in PBS and subsequently washed twice in PBS for 10 min. β-Galactosidase activity staining was performed as described by Mismer and Rubin (1987).

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