GENETIC ANALYSIS OF DROSOPHILA LARVAL OPTIC NERVE DEVELOPMENT

Amy L. Holmes, R. Nathan Raper and Joseph S. Heilig

Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, Colorado 80309-0347

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ABSTRACT

To identify genes necessary for establishing connections in the Drosophila sensory nervous system, we designed a screen for mutations affecting development of the larval visual system. The larval visual system has a simple and stereotypic morphology, can be recognized histologically by a variety of techniques, and is unnecessary for viability. Therefore, it provides an opportunity to identify genes involved in all stages of development of a simple, specific neuronal connection. By direct observation of the larval visual system in mutant embryos, we identified 24 mutations affecting its development; 13 of these are larval visual system-specific. These 13 mutations can be grouped phenotypically into five classes based on their effects on location, path or morphology of the larval visual system nerves and organs. These mutants and phenotypic classifications provide a context for further analysis of neuronal development, pathfinding and target recognition.

DEVELOPMENT of a complex nervous system requires establishing and maintaining precise connections between sensory organs and the brain. These connections are often established during early embryogenesis and are maintained in spite of drastic morphological changes that occur during development. Specific connections between peripheral organs and the brain must be established amidst a complex network of neuronal connections. Establishment of neuronal connections is dependent upon axons finding their proper path, recognizing their target, and forming synapses. In the course of establishing its connections, an axon often grows over a large number and variety of cells from which it must select the appropriate target. The processes of growth and target selection require coordination of cytoskeletal rearrangements (reviewed in Tanaka and Sabry 1995), cell adhesion, and intercellular communication (reviewed in Goodman and Shatz 1993). These processes allow the growing nerve to recognize and respond to directional signals along its path (Bastiani et al. 1985; Goodman and Shatz 1993).

Although in many cases neuronal pathfinding is well described anatomically, it is poorly understood mechanistically. Mechanisms proposed to account for neuronal guidance in several organisms include: the blueprint hypothesis (Singer et al. 1979), in which nerve growth is directed by spaces in the tissue to be innervated, for example, innervation of Drosophila wing veins (Palka et al. 1983); the guidepost or stepping-stone model (Bate 1976), in which a nerve contacts a series of cells distributed along its path, as in growth of the pioneer neurons in the grasshopper leg (Bentley and Keshishian 1982a,b); and the chemoaaffinity hypothesis elaborated by Sperry (1963), which predicts the existence of diffusible molecules secreted by a target that act from a distance to attract specific nerves. Variations and combinations of these hypotheses will almost certainly be necessary to accurately describe pathfinding.

Identification of similar molecules in vertebrates and invertebrates reveals that at least some neuronal pathfinding mechanisms have been maintained throughout evolution. Many of these molecules have been identified by in vitro analyses of pathfinding (for example, Bonhoeffer and Huf 1985; Luo et al. 1993; Serafi et al. 1994; Colamarino and Tessier-Lavigne 1995; Drescher et al. 1995), and many have subsequently been shown to be expressed in vivo in patterns consistent with roles in axonal guidance (Kennedy et al. 1994; Cheng et al. 1995; Luo et al. 1995). These studies eventually led to the identification of the genes encoding the molecules active in vitro (Bier et al. 1989; Chiba et al. 1995). Direct demonstration that the pathfinding genes identified in vitro are involved during normal in vivo development in axonal growth and guidance has been less forthcoming. Recently, genetic screens for defects in central nervous system (CNS) and motoneuron development in Drosophila have led to identification of genes involved in neuronal pathfinding (Seeger et al. 1993; Van Vactor et al. 1993). A screen for mutations affecting innervation of the adult optic lobes also led to recovery of genes required for establishment of neuronal connections (Martin et al. 1995). We designed and conducted a screen for mutations on the first and second chromosomes that disrupt the larval visual system (LVS) of Drosophila melanogaster. A similar approach to identify mutations on the third chromosome that dis-
rupt the LVS was recently reported by Schmucker et al. (1997).

Development of the LVS has been well described (Tix et al. 1989; Schmucker et al. 1992; Green et al. 1993; Schmucker et al. 1997) and is summarized in results. The photosensory organs and nerves have a characteristic morphology, which allows easy recognition of disruptions in development of the LVS. Although neuronal development often involves coordinated expression of many genes, some with redundant function, several studies have shown that the LVS can be disrupted by mutations in single genes (Steller et al. 1987; Moses et al. 1989; Heilig et al. 1991; Schmucker et al. 1992; Cheyette et al. 1994; Schmucker et al. 1994, 1997). However, of those characterized molecularly, all but one of these genes encodes transcription factors and thus are not directly involved in the intercellular events necessary for establishment of neuronal connections.

We identified 24 recessive mutations that disrupt the LVS. Defects resulting from these mutations include improper placement of the photosensory organs, abnormal axonal pathways, bifurcation or defasciculation of the nerve, and abnormal morphology of the nerve and photosensory organs. We have also recovered mutations affecting establishment of the proper number of larval photoreceptor cells. The phenotypes resulting from the mutations we recovered may be explained by disruptions in a variety of genes, including genes involved in fasciculation, pathfinding, migration and target recognition. We also identified mutations that appear to affect development of the LVS secondarily. For example, mutations causing disruption of head involution, or head morphogenesis, may lead to disruptions in the LVS, since the final position of the larval photosensory organs is likely to be influenced by head involution (Schmucker et al. 1992). Here we present genetic and phenotypic analyses of a subset of the mutants we identified whose primary phenotypes are defective development of the LVS.

**MATERIALS AND METHODS**

**Mutagenesis and screening:** To identify mutations on the second chromosome, 1-4-day-old male flies homozygous for bw were mutagenized with 25 mM ethylmethane sulphonate (Lewis and Bacher 1988). Mutagenized males were crossed en masse to g P[gl-lacZ]; Sco/ CyO, P[dav-lacZ] females. Individual F1 males of the genotype P[gl-lacZ]; bw */ CyO, P[dav-lacZ] (where * indicates a mutation) were backcrossed to establish independent lines carrying a single mutagenized second chromosome balanced with CyO, P[dav-lacZ]. To identify mutations, 50-200 1-4-day-old flies were put into aerated 50-ml conical tubes with standard egg collection media in the caps and set upside down for egg collecting. After 24 hr of acclimation, 0-3 hr embryos were collected and aged for 12 hr. The embryos were immersed in X-gal staining solution 7.2 mM NaHPO4, 2.8 mM Na2PO4, 150 mM NaCl, 1 mM MgCl2, 3 mM K[FeII(CN)6], 3 mM K[FeIII(CN)6], 0.3% Triton X-100, 0.2% X-gal} overnight at 37° (Lis et al. 1983; Simon et al. 1985).

For antibody staining, embryos were collected, dechorionated, fixed in 1:1 formaldehyde:heptane for 10 min while shaking. The embryos were rinsed in water, then washed in 1× PBT for a minimum of 4 hr, with 8 changes of PBT. The embryos were immersed in X-gal staining solution 7.2 mM NaHPO4, 2.8 mM Na2PO4, 150 mM NaCl, 1 mM MgCl2, 3 mM K[FeII(CN)6], 3 mM K[FeIII(CN)6], 0.3% Triton X-100, 0.2% X-gal} overnight at 37° (Lis et al. 1983; Simon et al. 1985).

**RESULTS**

**Identification of mutants with disruptions in the larval visual system:** To identify genes necessary for development of the LVS, we designed a simple histological screen that allowed us to identify mutant embryos by direct examination of the LVS. We established independent mutagenized stocks carrying a P[gl-lacZ] reporter construct (Moses and Rubin 1991) that directs lacZ expression to the photoreceptor cells. This permits easy identification of the LVS in staged embryos; a “blue balancer” (Bell et al. 1989) distinguished homozygous and heterozygous embryos (see Materials and Methods for details of screen). To identify mutations affecting any stage of LVS development, we examined X-gal-stained gl-lacZ embryos 12-15 hr after egg laying (AEL), at which point the LVS is fully developed in wild-type embryos (Figure 1, C and D). The screen was designed to allow identification of mutations affecting the LVS, even if they were associated with a postembryonic lethal phenotype, and to screen multiple embryos homozygous for the mutagenized chromosome were identified by the absence of dav-lacZ staining. We examined 1935 independent mutagenized lines and scored 1401 of these lines, in which we could see gl-lacZ expression, for second chromosome mutations causing LVS defects.

The X chromosome was screened in a similar manner. Four-day-old g P[gl-lacZ] males were mutagenized with ethylmethane sulphonate and mated to d(1)H A32/FM 7c, P[ftz-lacZ] females. Individual F1 females of the genotype, P[gl-lacZ]/ FM 7c, P[ftz-lacZ] were backcrossed to FM 7c, P[ftz-lacZ] males. Embryos were collected and scored as described above. We examined 1853 independent mutagenized lines and scored 1578 for X chromosome mutations causing LVS defects.

Initial genetic localization of the mutations was determined using the appropriate set of deficiencies from the Bloomington Stock Center (Bloomington, IN).

**Histology:** β-galactosidase activity was detected by collecting embryos in 1× PBT (1× PBS, 0.5% Triton X-100), dechorionating in 50% bleach for 4 min, and rinsing thoroughly in water, followed by fixation in 2.5% glutaraldehyde-saturated heptane for 10 min while shaking. The embryos were rinsed in water, then washed in 1× PBT for a minimum of 4 hr, with 8 changes of PBT. The embryos were immersed in X-gal staining solution 7.2 mM NaHPO4, 2.8 mM Na2PO4, 150 mM NaCl, 1 mM MgCl2, 3 mM K[FeII(CN)6], 3 mM K[FeIII(CN)6], 0.3% Triton X-100, 0.2% X-gal} overnight at 37° (Lis et al. 1983; Simon et al. 1985).

For antibody staining, embryos were collected, dechorionated, fixed in 1:1 formaldehyde:heptane for 20 min, and devitellinized by shaking in 1:1 heptane:methanol for 1 min (Mitchison and Sedat 1983). Concentrations of primary antibodies were used: MAb 22C10, 1:100 (gift of L. Zipurisky) (Fujita et al. 1982; Zipurisky et al. 1984); anti-β-galactosidase, 1:500 (Promega, Madison, WI); anti-HRP, 1:100 (Cappel, Durham, NC) (Jan and Jan 1982). The goat-anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (Bio-Rad, Richmond, CA) was preabsorbed against 12-15 hr-old embryos and used at 1:500. HRP-conjugated secondary antibodies were visualized using 0.5 μg/ml DAB + 0.003% H2O2, Embryos were dehydrated through an ethanol series, mounted in 1:3 methyl salicylate:Permount and examined under Nomarski optics.

**RESULTS**

**Identification of mutants with disruptions in the larval visual system:** To identify genes necessary for development of the LVS, we designed a simple histological screen that allowed us to identify mutant embryos by direct examination of the LVS. We established independent mutagenized stocks carrying a P[gl-lacZ] reporter construct (Moses and Rubin 1991) that directs lacZ expression to the photoreceptor cells. This permits easy identification of the LVS in staged embryos; a “blue balancer” (Bell et al. 1989) distinguished homozygous and heterozygous embryos (see Materials and Methods for details of screen). To identify mutations affecting any stage of LVS development, we examined X-gal-stained gl-lacZ embryos 12-15 hr after egg laying (AEL), at which point the LVS is fully developed in wild-type embryos (Figure 1, C and D). The screen was designed to allow identification of mutations affecting the LVS, even if they were associated with a postembryonic lethal phenotype, and to screen multiple embryos...
Figure 1.—Normal development of the larval visual system. (A) Dorsal view of stage 13 wild-type embryo. BO (arrowheads) are ectodermal and have not yet begun to migrate anteriorly. (B) Lateral view of stage 14 embryo. BO (arrowhead) has begun anterior migration and the BN (arrow) can be seen extending from it toward the brain. The other BO and BN are out of the plane of focus. (C) Dorsal view of stage 16 embryo. BOs (arrowheads) have reached their final anterior position and BNs have assumed their final position in the embryo. Because the nerve makes a ventral turn (arrows), only part of the nerve is in the plane of focus. At stage 16, there are also gl-lacZ expressing cells in the brain. The gl-lacZ-expressing tissue between the BOs is an unknown tissue (Moses and Rubin 1991). (D) Lateral view of stage 16 embryo. The entire trajectory of one BN from BO (arrowheads) to brain can be seen. The BN turns ventrally where it reaches the anterior of the brain (arrow). (E) Schematic of stage 16 embryo, dorsal view. BOs and location of the brain are indicated. Unidentified tissue expressing gl-lacZ is indicated with an asterisk. (F) Schematic of stage 16 embryo, lateral view. BO, BN and brain are indicated. In all figures, anterior is to the left; dorsal is towards top in lateral views. Wild-type embryos carrying gl-lacZ were fixed and stained with anti-β-galactosidase as described in materials and methods.

From each mutagenized stock for reproducible defects. After initial identification, mutant embryos were retested by X-gal staining, and those exhibiting a reproducible LVS defect were stained with an antibody recognizing β-galactosidase (β-gal) for higher resolution analysis of the mutant phenotype.

We identified 11 mutations on the second chromosome and 13 mutations on the X chromosome that result in disrupted development of the LVS. All of these mutations are recessive and both complementation analysis and initial mapping studies suggest that each represents a unique gene. Although the complementation data suggest we have recovered single alleles of each gene, we have analyzed the phenotypes resulting from each mutation when hemizygous with a deletion that uncovers the phenotype. In each case, the phenotype resulting from the mutation when hemizygous resembles the phenotype when homozygous, indicating we have recovered amorphic or hypomorphic alleles. Deletions defining the genomic location of each mutation presented here are listed in Table 1.

To study further the mutants specifically disrupted in development of the LVS, we inspected the overall morphology and the embryonic development of both the central nervous system (CNS) and the peripheral nervous system (PNS) to distinguish mutations causing
### TABLE 1

Phenotypes associated with mutations disrupting the larval visual system

<table>
<thead>
<tr>
<th>Mutation/gene</th>
<th>Percent expressivity of LVS phenotype</th>
<th>Percent larval visual system defect&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Non-comp Df (Df breakpoints)</th>
<th>Viable&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CNS&lt;sup&gt;c&lt;/sup&gt;</th>
<th>PNS&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>dalm</td>
<td>100</td>
<td>100</td>
<td>Df(2R)Pd11B 55A1;55C1-3</td>
<td>−</td>
<td>ND</td>
<td>−</td>
</tr>
<tr>
<td>sine</td>
<td>82</td>
<td>74</td>
<td>Df(2)TW161 38A6B1;40A4-B1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>roam</td>
<td>83</td>
<td>60</td>
<td>Df(1)g 11F10;12F1</td>
<td>+</td>
<td>ND</td>
<td>−</td>
</tr>
<tr>
<td>ramtl</td>
<td>71</td>
<td>51</td>
<td>Df(2)ST1 43B3-5;43E15-18</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>knax</td>
<td>78</td>
<td>60</td>
<td>Df(1)A27 18A5;18D</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>jok</td>
<td>74</td>
<td>127</td>
<td>Df(1)A27 18A5;18D</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>oop</td>
<td>71</td>
<td>90</td>
<td>Df(1)A113 3D6E1;4F5</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>nate</td>
<td>78</td>
<td>76</td>
<td>Df(1)A209 20A,20F</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>tric</td>
<td>67</td>
<td>161</td>
<td>Df(2)N10 35E1-2;36A6-7</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup> Defects of each phenotype are given as a percentage of those embryos that express a larval visual system phenotype (first column). Phenotypes never seen are recorded as −.

<sup>b</sup> Mutations are scored as + if they are homozygous viable or viable over a deficiency that uncovers the larval visual system phenotype.

<sup>c</sup> Mutations are scored as + if they have no detectable defect and as − if they do have a detectable defect.

ND = not determined.

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n = number of embryos scored.
general developmental or neuronal defects from those specifically affecting the LVS. As summarized in Table 1, all of the mutants we selected for future study were evaluated for viability, embryonic morphology, CNS development [using an antibody to HRP (Jan and Jan 1982)], and PNS development [using the monoclonal antibody 22C10 (Fujita et al. 1982; Zipursky et al. 1984)]. Our analysis of the PNS and CNS was limited to detecting gross abnormalities so that subtle defects may have gone undetected. From these initial analyses we concluded that 10 of the 24 mutations affect head or embryonic morphology. We focused our analysis on 13 mutant phenotypes that predominantly affect LVS development, and one that affects establishment of photoreceptor cell fate; initial phenotypic characterization of a subset of these mutants is summarized in Table 1.

**Development of the larval visual system:** In wild-type embryos, the LVS consists of two photosensory organs identified by Bolwig (1946) in the larvae of Musca and thus called the Bolwig's organs (BOs). Each BO is composed of 12 neurons, located on either side of the midline in the anterior of the larva (Figure 1, arrowheads). The BOs are derived from cells located adjacent to the optic placode, which gives rise to the adult optic lobe (Green et al. 1993). The BOs are first detectable at stage 12 of embryonic development by expression of Krüppel protein (Schmucker et al. 1992; Schmucker et al. 1997). We first detect gl-lacZ expression shortly after Krüppel is expressed, during late embryonic stage 12 (Figure 1A), which corresponds to nine hours after egg laying (AEL) (all stages as described in Campos-Ortega and Hartenstein 1985). Development of the BO is first detected when a single cell on each side of the embryo begins expressing Krüppel. Shortly thereafter, this single photoreceptor cell extends an axon toward the brain (Schmucker et al. 1992). Following establishment of the pioneer connection, additional cells become committed to the photoreceptor cell fate and extend axons that fasciculate with the pioneer axon, forming the Bolwig's nerve (BN) (Schmucker et al. 1992). The 12 cells forming the BOs are clustered by stage 13 (~10 hr AEL) (Figure 1A, arrowheads), by which time the axons from at least four of the photoreceptor cells have established connections with the developing brain (Green et al. 1993; Schmucker et al. 1997).

During head involution (stage 14 or 10–11 hr AEL), the BOs move anteriorly and the BNs increase in length (Figure 1B) (Steller et al. 1987; Schmucker et al. 1992; Green et al. 1993; Schmucker et al. 1997). Green and colleagues (Green et al. 1993) proposed that the initial connections between the larval photosensory axons and the brain are maintained for the remainder of development. The mature LVS is established by completion of head involution, at which time the BOs have assumed the positions they will maintain for the duration of embryonic development (Figure 1, C and D). The projections of the BNs are characterized by a sharp turn where

**Figure 2.—** dalm affects photoreceptor fate. Lateral view of stage 13 dalm- embryo. Cells expressing gl-lacZ are located throughout the embryo, rather than in their normally restricted pattern (compare to Figure 1B). dalm- embryos carrying gl-lacZ were fixed and stained with anti-β-galactosidase.

**Figure 3.—** sine- disrupts BN and BO morphology and BN trajectory. (A) Dorsal and (B) lateral views. In sine- embryos the morphology of the LVS is generally normal, as the BNs follow the proper path and terminate appropriately and the BOs are in the proper location. However, BOs (arrowheads) are larger than in wild type and BNs (arrows) are thicker and wavier. Stage 16 sine- embryos carrying gl-lacZ were fixed and stained with anti-β-galactosidase. The anterior regions of two different sine- embryos are shown.
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**Figure 5.** roam\(^{-}\) disrupts PNS development in a subset of mutant embryos. (A) dorsal view of wild-type embryo stained with MAb 22C10. Note ordered appearance of ventral nerve cord (arrow) and nerves of the peripheral nervous system (arrowhead). (B) dorsal view of a roam\(^{-}\) mutant embryo stained with MAb 22C10. Note the disrupted appearance of the ventral nerve cord (arrow) and the nerves of the PNS (arrowheads).

number of embryonic cells to initiate a photoreceptor cell fate. In dalm\(^{-}\) embryos, ectopic gl-lacZ expression is detected throughout the embryo (Figure 2). When dalm\(^{-}\) embryos are stained with MAb 22C10, which recognizes a PNS-specific antigen (Fujita et al. 1982; Zipursky et al. 1984), the pattern of expression resembles that of gl-lacZ, indicating that ectopic gl-lacZ expressing cells in dalm\(^{-}\) embryos have initiated neuronal development (data not shown). The similar expression of neuronal and photoreceptor-specific markers at ectopic sites throughout dalm\(^{-}\) embryos indicates that the dalm\(^{-}\) mutation causes cells not normally destined to become photoreceptor cells to assume a photoreceptor cell fate. Embryos homozygous for the dalm\(^{-}\) mutation do not survive past embryonic stage 13. We do not yet know whether dalm\(^{-}\) disrupts other developmental processes in addition to photoreceptor development.

**Mutation affecting BN trajectory:** The phenotype of sine\(^{-}\) mutant embryos is unique to those identified in our screen in that the trajectory of the BNs is abnormal even though the BNs follow a normal path and appear to terminate normally (Figure 3, A and B, arrows). The BNs are much thicker than normal (57% of mutant embryos) (Figure 3, A and B, arrowheads). The large BO phenotype as well as the thick BN phenotype may result from greater numbers of photoreceptor cells or from photoreceptor clusters that are more loosely associated than normal.

**Mutation affecting BN path and morphology:** In three mutant strains, including roaming\(^{-}\) (roam\(^{-}\)) and rambling\(^{-}\) (rambl\(^{-}\)), the primary defect is disruption of the normal path and morphology of the BNs. Examples of the roaming\(^{-}\) phenotype are shown in Figure 4. The BN path is abnormal in a subset of roaming\(^{-}\) embryos (compare Figure 4 to Figure 1C, arrows). In 22% of roaming\(^{-}\) embryos, the BNs do not reach their normal place of termination (Figure 4, A–D, arrows). In some roaming\(^{-}\) embryos, the

**Description of the mutant phenotypes:** Based on initial phenotypic analyses, we grouped the mutations affecting LVS development into five phenotypic classes. Representatives of each class are described below. Because many of the mutants display a range of phenotypes overlapping several classes, the mutants are classified by their predominant phenotypes (Table 1).}

**Figure 4.** roam\(^{-}\) disrupts BN path and morphology. A range of morphological defects is seen in the BNs of roam\(^{-}\) embryos. (A) is lateral view, (B–F) are dorsal views. (A) BN extends posteriorly but then turns anteriorly terminating in loop (arrow). (B) BOs are elongated (arrowheads), BN is significantly thicker than normal (double arrow) and follows an abnormal path. BN terminates prematurely (arrow). (C) BO has not migrated to its proper position (arrowhead) and BN extends beyond its normal target (arrow). (D) Trajectory of BNs is abnormal (double arrows), turning toward the midline rather than away from the midline as wild-type BNs do. At least one BN (arrow) extends beyond its normal target. (E) Higher magnification view of different embryo revealing bifurcation of BN and nodules extending from it (double arrows). Arrowhead indicates BO. (F) Bifurcation of BN (arrow) in stage 14 embryo when the BO (arrowhead) has just begun anterior migration. roam\(^{-}\) embryos carrying gl-lacZ were fixed and stained with anti-\(\beta\)-galactosidase. All embryos are stage 16 except panel F which is stage 14.

establishment of the proper BN path and the proper position of the BOs.

**Mutation affecting photoreceptor cell fate:** One mutant strain, dalmatian\(^{-}\) (dalm\(^{-}\)), appears to cause an excessive
BNs terminate much further anteriorly than normal (Figure 4, A and B, arrows), whereas in others the BNs extend beyond their normal target (Figure 4, C and D, arrows). An example of early termination of the BN is shown in Figure 4A, where the nerve terminates unusually in a large loop and appears to be poorly fasciculated. Another example of the aberrant BN path phenotype of roam\(^{−}\) mutant embryos is shown in Figure 4D (double arrows). In this example, the BNs appear to extend normally from the BO to the sharp turn; however, after this point they turn toward the midline, unlike wild-type embryos, in which the nerve turns away from the midline (compare Figure 4D to Figure 1C). BN terminations in roam\(^{−}\) embryos are sometimes much closer to the midline than wild-type terminations (Figure 4D, arrow, compare to Figure 1C). In 14% of roam\(^{−}\) embryos, one or both of the organs does not reach its normal anterior position (Figure 4C, arrowhead).

BN morphology is abnormal in 68% of roam\(^{−}\) embryos. The embryos have either an abnormally thick BN (Figure 4B, double arrows) or swollen areas (nodules) along the length of the BN (Figure 4E, double arrows). Aberrations in BN morphology are apparent early in development of roam\(^{−}\) embryos. Bifurcations of the BN are already visible at embryonic stage 14, when the BOs are still close to the brain (Figure 4F, arrow).

Although roam\(^{−}\) animals are viable, suggesting that when homozygous the mutation does not cause severe developmental or neuronal defects, analysis of roam\(^{−}\) embryos using MAb 22C10 and anti-HRP reveals abnormalities in PNS and CNS development. In 20% of roam\(^{−}\) embryos, the nerves extending from the ventral nerve cord are disorganized (compare Figure 5, A and B). We have not further characterized the PNS and CNS defects in roam\(^{−}\) embryos. The phenotype of a second member of this class, rambling, is presented in Table 1 and will be described in detail elsewhere (A. L. Holmes and J. S. Heilig, unpublished results).

Mutations affecting BN morphology: In four mutant strains the BO migrates normally and the overall path of the BN appears normal. The primary defect is the presence of nodules along the BN. Although most of the mutants presented in this report have defects in nerve morphology (Table 1), mutants of this class exhibit few other LVS phenotypes. Representatives of this class are knobby axons (knax) (Figure 6) and just odd knobs (jok) (Figure 7). Nodules are present in 87% of knax\(^{−}\) embryos (Figure 6, A and B, arrows) and 60% of

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Figure 6.—knax\(^{−}\) disrupts BN morphology. (A, C) dorsal; (B) lateral views. (A) Nodules are present in both nerves at the position of the ventral turn (arrows). (B) BN (arrow) morphology is abnormal, with a series of nodules along the length of the nerve. BO is indicated (arrowhead). (C) BN bifurcates (arrow) at ventral turn. Stage 16 knax\(^{−}\) embryos carrying gl-lacZ were fixed and stained with anti-\(β\)-galactosidase.
jok- embryos (Figure 7A, arrow); bifurcations are present in 17% of knax- embryos (Figure 6C, arrow) and 14% of jok- embryos (Figure 7B, arrow). Nodules and bifurcations are most often present at the turn of the BN (Figure 6, A and C; Figure 7, A and B, arrows), although they do appear elsewhere along the BN path (Figures 6B and 7C, arrows). Bifurcations in the BN are seen infrequently near the BO, and bifurcating axons sometimes terminate in small nodules (Figure 7C, arrow). Despite the abnormal morphology of the BN, the path is approximately normal (compare Figures 6 and 7 to Figure 1, C and D).

The nodules seen along the BN in knax-, jok- and other mutant embryos may result from either misplaced BO cell bodies or defects in axon morphology. To distinguish between these possibilities we used antibody to Glass protein, a nuclear protein present in all photoreceptor cells (Moses et al. 1989) to determine whether photoreceptor nuclei were present in nodules. In jok- embryos Glass protein is present in the nodules (not shown), suggesting that the nodules in jok- embryos result from misplaced photoreceptor cell bodies.

Mutations affecting morphology of BN and organs: In five of the mutant strains the primary defect is in the shape and placement of the BOs. The BOs in greater than 25% of embryos homozygous for either out of place- (oop-) (Figure 8A) or not enough anterior extension- (nate-) (Figure 8, B-D) are either misplaced (Figure 8, A and B, arrowheads) or misshapen (Figure 8B, double arrowheads). Interestingly, in a subset of oop- embryos in which a BO does not reach the proper place, a few photoreceptors appear to have separated from the major cluster and migrated to the correct location (Figure 8A, asterisk). The group of photoreceptor cells that successfully migrated to the normal position (compare Figure 8A to Figure 1C) typically contains a minority of the photoreceptor cells in that side of the embryo. Axons extending from the smaller cluster appear to extend toward and fasciculate with the major bundle of axons from the misplaced BO. Strikingly, the BOs that stop migrating prematurely almost always turn toward the midline of the embryo (Figure 8A, arrow; see also Figure 4C, arrowhead).

Although the BO defects are the unique characteristic of this group of mutants, most also display defects in BN morphology. The frequency of BN defects is greater in nate- (approximately 80%) in contrast to oop- (about 25%). The most common BN defects are bifurcations (Figure 8C, arrow) or nodules (Figure 8C, double arrowhead) and, although these defects can be seen as early as stage 14 (Figure 8D, arrow), the BNs appear to reach the brain normally in most mutant embryos (Figure 8, B and C, double arrows; compare to Figure 1C).

We mapped oop- to a deletion that also uncovers fasciclin II (fasII), a gene with a demonstrated role in motoneuron development (Grenningloh et al. 1991; Lin et al. 1994). By examining the LVS of gl-lacZ embryos...
transheterozygous for opo and fasII^{66}, we determined that opo and fasII^{66} complement each other, indicating the opo mutation does not affect the fasII gene, but affects a neighboring locus.

We isolated three other members of this class, and the predominant defect in one, triclops (tric) (Table 1), is in enumeration or adhesion of the larval photoreceptor cells. In a subset of tric embryos, three distinct BOs are seen. Further analysis of tric will be presented elsewhere (A. L. Holmes and J. S. Heilig, unpublished results).

Mutations disrupting LVS do not have an adult visual system phenotype. To address the question of dependence of adult visual system development on LVS development (reviewed in Meinertzhagen and Hanson 1993), we analyzed adult visual system development in flies homozgyous for the mutations described above. None of the 13 mutations we recovered that specifically disrupt the LVS are lethal and none appear to affect compound eye development (not shown). The BN has been proposed to be required for optic stalk development and subsequent innervation of the adult optic lobe by the adult photoreceptor axons (Steller et al. 1987). To determine whether the optic stalk develops normally in these mutants, and whether the BN projects through the optic stalk, we examined the connection between the eye-antennal imaginal disc and brain in third instar larvae homozgyous for mutations in sine, roam, knax, and jok. Interestingly, our preliminary analysis reveals that for all of these mutants, although the BNs in third instar larvae are abnormal, at least some BN axons appear to enter the optic stalk. The abnormalities we see in the BNs of these larvae include apparently defasciculated BNs, axons terminating prematurely in the disc, and abnormally thin BNs.

### SUMMARY AND DISCUSSION

We have identified 24 mutations causing a range of mutant phenotypes in the LVS. The phenotypes of some of the mutants we recovered are similar to the phenotypes associated with mutations in other genes known to have a role in LVS development. For example, mutations in disco result in BNs that do not maintain association with their targets in the brain and bifurcate and terminate in abnormal locations (Steller et al. 1987). This phenotype is similar to that of roam^+ mutant embryos. Mutations in Krüppel lead to misshapen BOs and bifurcations of the BN (Schmucker et al. 1992), similar to the phenotype of nate^+ embryos. Schmucker et al. (1997) identified mutations in 13 genes on the third chromosome which disrupt the LVS. The resulting phenotypes of these mutations were either disruption of the BN, as is seen in rambl^-, knax^-, and jok^-, or in disruption of the BO and BN, as is seen in opo^-, nate^-, and tric^-. Because of differences in analysis performed by Schmucker et al. (1997), it is difficult to place the mutations identified on the third chromosome (Schmucker et al. 1997) into the framework in which we analyzed our mutations. Many of the mutants identified by the two screens have similar LVS phenotypes, indicating the genes identified are likely to play roles in similar developmental steps. However, recovery of single alleles and phenotypes unique to each screen suggests that there are as yet unidentified loci involved in LVS development.

Described here are seven mutants, representing five phenotypic classes. The phenotypic classifications are defined by primary defects in the morphology or placement of the BN or BO. Mutants were classified by their predominant and most characteristic phenotypes but may exhibit defects overlapping those of other classes. With one exception not discussed here, none of the mutations we identified correspond to genes with known roles in neuronal development. This may reflect...
The LVS phenotype of ptc mutants is similar to that of sine embryos; the BOs are increased in size and the BNs are thick and poorly fasciculated. Since ptc is normally expressed in the optic lobe placode, which remains epithelial until late second instar, and not in the BO, Schmucker et al. (1994) proposed that ptc is responsible for suppressing neuronal fate in the optic lobe placode, similar to the role of Notch in the central nervous system. sine may play a role in the same pathway in which ptc acts or in a similar pathway to suppress neuronal cell fate in the optic lobe.

Extension of axons and establishment of connections: After establishment and differentiation of the photoreceptor cells of the BO, the photoreceptor cells extend axons which establish connections with the brain. If the initial connection is not made before the BO moves anteriorly, the unconnected end of the BN may be pulled along with the BO as it migrates. Once removed from proximity to the brain, the BN may be unable to follow the cues that normally guide the axons to the brain or these cues may no longer be available. As a consequence, the BN axons would grow in random paths, such as those characteristic of roam embryos. Although in roam embryos the BN path appears to be random, the axons almost always extend posteriorly, perhaps reflecting the established polarity of the photoreceptor cells in the BO.

Fasciculation of the BN: Following establishment of the pioneer connection, the axons extending from the other photoreceptors in the BO fasciculate with the pioneer and presumably follow its path to the brain. This process requires recognition of the pioneer and fasciculation with it coincident with axon extension. Although it is known that at least four photoreceptor cells in the BO extend axons early in LVS development, it has not been determined when the other eight photoreceptor cells present in the BO extend their axons. If these axons are extended later in development and use the original axons as guides, it is possible that the failure of axons extending from the BO to fasciculate and grow along the pioneer could result in the bifurcations and nodules such as those seen in knax−, jok−, roam−, oop−, and nate−, which have defects in BN morphology. Nodules could result from tangling of axons that have lost adhesion to the BN. These genes may be involved in any of a number of events required for proper fasciculation of the nerve. For example, several cell adhesion molecules have demonstrated roles...
in axon fasciculation (see, for example, Jay and Keshishian 1990; Lin et al. 1994; Chiba et al. 1995).

Anterior movement of the BO: During head involution, the BO moves anteriorly and the BN elongates to accommodate this movement. Whether the movement of the BO is an active process or whether the BO is swept along with the general displacement of head tissues is unknown. However, our recovery of mutations disrupting BO placement in embryos that appear to have undergone normal head involution suggests that BO movement can be genetically separated from the process of head involution. Therefore, it is likely that BO migration is an active process, not simply a consequence of head involution.

BO migration may require recognition of signals that direct the BOs to their correct final positions. Mutations disrupting a cue guiding BO migration may result in the BO’s movement, characteristic of oop- and nate- embryos. Failure of BO migration could result from mutations affecting temporary adhesion of the BO to recognition points along its path. Mutations affecting molecules involved in releasing temporary adhesion would delay or prevent the BOs from completing their anterior migration. Retarding migration of all or a subset of photoreceptors could cause the BO to assume the aberrant oblong shape seen in nate- embryos. It is especially interesting that BOs which fail to migrate in these mutant embryos almost always turn toward the midline and stall in the same apparent location. It will be important to determine if this position reflects the location of a structure involved in directing BO migration. Mutations resulting in disruption of BO migration may affect the ability of the BO to dissociate from a recognition point present at this location. Molecules involved in release from a recognition point may include anti-adhesion molecules that interact with cell adhesion molecules, disrupting the adhesion. A similar anti-adhesion role has been proposed for beater path, which has recently been shown to genetically interact with cell adhesion molecules to allow proper defasciculation in motoneurons (Fambrough and Goodman 1996).

The nodules observed in knax-, jok-, roam-, nate-, and oop- could also result from mutations causing disruption of migration of the BO. The photoreceptor-specific nuclear protein Glass is present in nodules in jok- embryos. The misplaced photoreceptor cell bodies present in jok- embryos, and perhaps in other mutant embryos, may result from a failure to dissociate from an adhesion point and become separated from the migrating BO.

Early in embryonic development, when the pioneer axons navigate their way to their targets, targets are close. Axons that extend later in development must travel longer distances through a more complex environment. Fambrough et al. (1996) proposed that kuzbanian (kuz), which encodes a zinc metalloprotease, is required for later-extending motoneuron axons to forage through a more complex environment than that through which the pioneer travels. Misplaced BOs and nodules could also result from mutations in genes that play a role similar to that of kuz in motoneuron development. The environment through which the developing BO must forage is also quite complex. Defects in proteases, or other molecules, required for the BO to migrate anteriorly through the developing head may result in the phenotypes observed in oop- and nate mutant embryos. Defects in proteins required for cytoskeletal rearrangements necessary for nerve growth (reviewed in Tanaka and Sabry 1995) could also explain the BO migration phenotype of oop- and nate-. Inability of the BN to grow would result in the BN acting as a tether preventing the BO from migrating normally.

As the BO moves anteriorly, it is possible that the BNs must also recognize signals necessary to assume their characteristic paths. These signals may be surface molecules on strategically positioned cells, either unique signals or those the BO also recognizes in its anterior migration. Mutations causing disruption of placement of these signal cells, of the signal molecules, or of the ability of the BN to recognize the signal would result in defective nerve paths such as those seen in roam- embryos. Bifurcations of the BN may reflect attempts of axons to find their positional signals.

Relationship between larval and adult visual systems: In addition to analyzing LVS development, the mutants we identified will allow us to address the dependence of adult compound eye development on proper development of the LVS (reviewed in Meinertzhagen and Hanson 1993). From their analysis of disco mutant embryos, Steller and colleagues proposed that maintenance of the connection between the BN and brain is necessary for formation of the optic stalk (Steller et al. 1987). In the absence of the optic stalk, no conduit exists to direct the adult photoreceptor axons to the developing optic lobes, and adult eye and optic lobe development are severely disrupted, a phenotype characteristic of disco mutant embryos (Steller et al. 1987; Heilig et al. 1991). Subsequent analysis of glass and other disruptions affecting LVS development (Moses et al. 1989; Kunes and Steller 1991) however, indicated that the optic stalk may form in embryos in which BNs are not seen. Resolution of the interdependence of development of the larval and adult visual organs has been hampered by the dearth of mutations affecting LVS development that do not otherwise affect development of the adult fly. Considering the severe disruptions in BN trajectory and morphology we see in mutant embryos it is striking that in all cases examined thus far, normal adult eye development is correlated with at least some BN axons having reached the brain and, perhaps, allowing development of the optic stalk. Higher resolution analysis of the BN, the optic stalk, and the adult visual system will reveal whether subtle disruptions of compound eye or optic lobe development result from these mutations. Overall, however, adult visual system
development appears normal when at least a subset of BN axons innervate the brain, reviving the question of the relationship between the larval and adult visual systems. The mutations identified in our screen lead to disruption of development of a specific sensory organ and its nerve. These mutations provide a means of specifically addressing development of the LVS as well as mechanisms of neuronal connectivity in general. For example, analysis of dalm and sine may demonstrate how the proper number of larval photoreceptor cells is established. The question of whether BO migration is an active or passive process may be revealed by analysis of op and nate. The BO and BN face developmental challenges common to nervous system development in general. For example, the BN must establish and maintain proper connections with the brain; roam may reveal how the BN makes its connection. Identification of cells acting as recognition points and molecules used to recognize these signals may result from analysis of knax and jok. Together, these mutations will allow us to dissect LVS development genetically and molecularly and enhance understanding of neuronal connectivity in general.

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