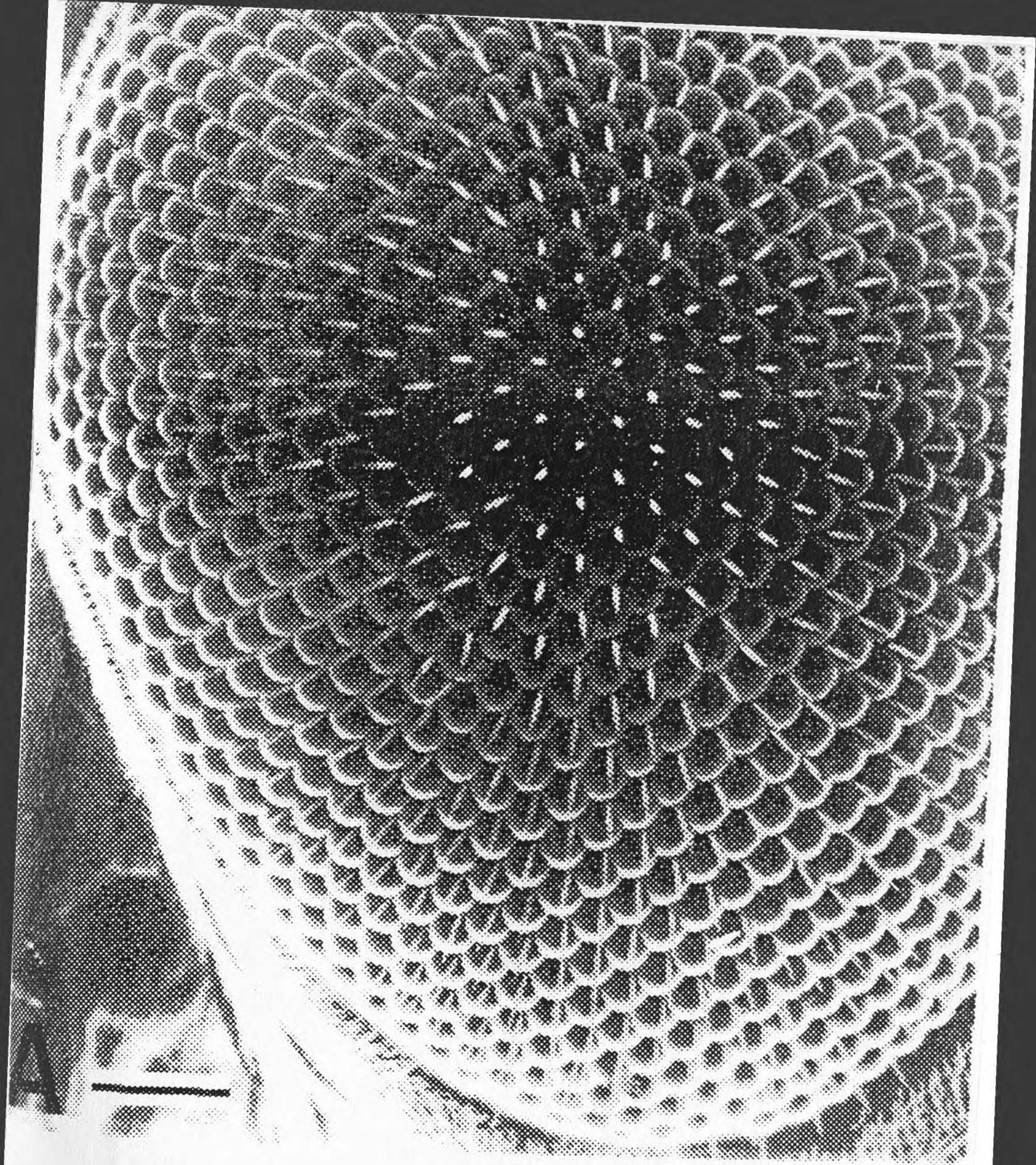

ig. 1. A. *Scanning electronmicrograph*

A *DROSOPHILA* (FRUIT FLY) VISUAL SYSTEM MUTANT: *GREENBLIND*

by

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A THESIS

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ABSTRACT

The nervous system of animals consists of diverse cell types in specific arrangements with precise and specific patterns of connections (synapses) with one another. The current view is that behaviors (defined and measurable action of an organism associated with central nervous system activity) are driven by the synaptic connections between groups of nerve cells (neurons). The human brain has 10^9 neurons each making on the average 1000 specific, non-random, connections with other neurons. It is not known how these connections are specified. The way in which this occurs is one of the central questions of neurobiology. Various evidence suggests that molecules acting as positional cues during development guide growing axons (the long cell extensions that interconnect neurons) to the appropriate target cells.

Genetics (the study of genes) is a useful method for investigating this problem since mutations can serve as a functional assay for proteins involved in synaptogenesis. A systematic search for behavioral mutants (in which the DNA sequence encoding a gene has been altered) of the jump reflex of *Drosophila* has been used to identify molecules involved in the specification of neural connections. The *Drosophila* visual system has a number of advantages as a system for studying behavioral mutants, and may be especially suited to a mutational study of synaptic

specificity.

We have identified a *Drosophila* behavioral mutant, *grb*, which has the properties expected of a neuronal connectivity mutant. *grb* Mutant flies are blind to green light but respond normally to UV light. *grb* flies have the full complement of photoreceptor neurons (light sensitive cells in the retina). Mutant photoreceptors have a normal electrophysiological response to light. This situation argues that the mutation specifically alters the neural connectivity between the photoreceptor cells mediating green light phototaxis (movement of an organism toward light) and their neuronal targets in the brain, and thus *that grb* may identify a molecule involved in specifying a subset of neural connections in the *Drosophila* visual system. A mutation of this type has not previously been identified.

The work presented here describes the isolation and initial characterization of *grb* and proposes additional studies to define further the role of the *grb* protein in eye development (a single cell producing a multicellular organism or organelle) and in the formation of neural connections between the retina and the optic lobes (the part of the brain that processes visual information). Central to this aim are the isolation of a null allele (a mutation in which the gene is completely inactivated) of *grb*, determining in which cells and during what time in development the *grb* product is required and comparing the

grb product with other molecules with known or suspected roles in synaptogenesis via the molecular cloning (isolation of the piece of DNA bearing the gene) of the gene.

INTRODUCTION

DROSOPHILA COMPOUND EYE

The compound eye is composed of 800 identical subunits called ommatidia. Each of these has 22 cells of a few types in a characteristic arrangement. In the center of each ommatidium is a cluster of 8 photoreceptor cells (R - cells). There are three types of R cells, R1 - R6, R7 and R8. These are distinguished genetically, by position, spectral sensitivity and axonal targets in the optic lobes. Cells R1 - R6 occupy the periphery of the cluster, express an opsin (the photosensitive molecule of photoreceptor cells) sensitive to green light and send their axons to the first optic lobe. Cell R8 occupies a proximal position in the center of the cluster, sends axons to the medulla and expresses an opsin sensitive to blue green light. Cell R7 occupies a distal position in the center of the cluster, expresses a UV sensitive opsin and sends an axon to the second optic lobe. The remaining cells are bristle cells (mechanosensory cells), cone cells (lens secreting cells) and pigment cells (pigments are light opaque substances;

pigment cells optically isolate the photoreceptor cells between ommatidia).

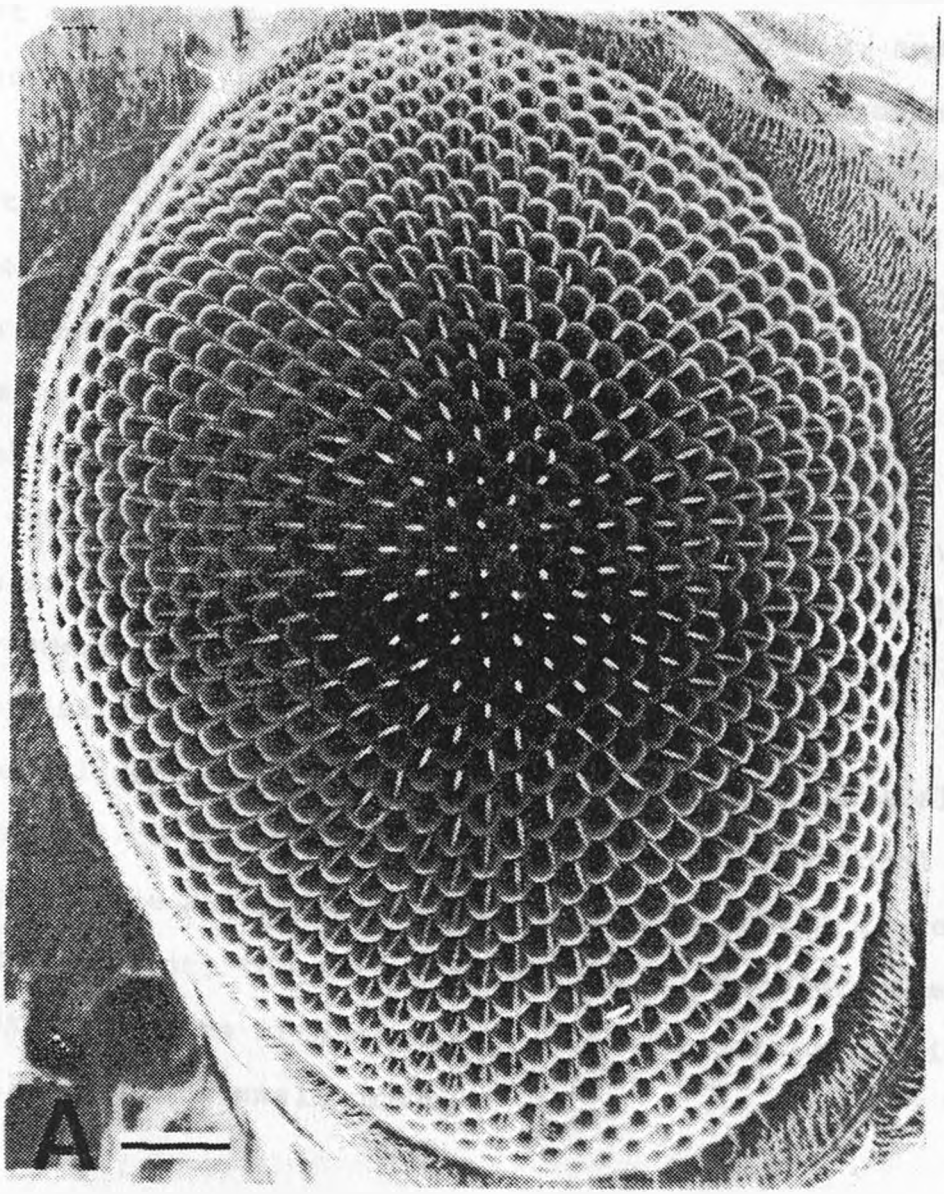


Figure 1:

Scanning electron micrograph of Drosophila compound eye showing repeated modular arrangement of ommatidia. Mechanosensory bristles extend from between the facets.

GENETIC STUDIES OF THE EYE

The visual system is ideal for genetic studies because it can be perturbed, or eliminated completely, and the flies are still viable (Harris, 1976). The visual system supports a variety of relatively simple and more complex behaviors. It is a suitable system for the genetic dissection of the nervous system on anatomical, behavioral and functional grounds. Visual system mutations have been isolated on the basis of morphology (Fischbach), electrophysiology (Pak) and behavior (Benzer). Compound eye mutations are easy to detect - over 200 are known (Lindsley and Grell, 1968). Individual cells can be identified by their position, and their movements and differentiation (maturation of cell including acquisition of biochemical and morphological specializations) followed (Tomlinson, 1988). This allows the analysis to be performed at the level of individual cells. Because the arrangement of cells in the retina is repeated and invariant, the retina serves as a magnifier of events that affect the arrangement or differentiation of single cells within a small group.

EYE DEVELOPMENT

The compound eye originates as a group of cells sequestered by an invagination of larval ectoderm. Cell

proliferation during larval development produces the compound eye primordium, the eye-antennal imaginal disc, a flat sheet of undifferentiated epithelial cells. Differentiation of cell types and pattern formation proceeds in an anterior to posterior fashion across the disc surface. A fold in the disc surface, the morphogenic furrow, marks the zone of most active cell differentiation. Behind the advancing furrow are arranged rows of cell clusters with fine axonal projections trailing into the brain (Zipursky and Venkatesh, 1985). Markers of differentiation, in addition to the furrow, are synchronized waves of cell division, the choreographed migration of cell nuclei vertically in the epithelium (Tomlinson, 1988), the successive appearance of specific antigens (Zipursky and Venkatesh, 1985), the formation of cell clusters and the incremental addition by pairs of cells to these clusters.

CHARACTERIZATION OF *GRB*

MUTAGENESIS AND ISOLATION

A hybrid dysgenic cross involves mating a 'P' cytotype male to an M cytotype female. 'P' type transposable elements are mobilized in the germline of the F1 (first generation) progeny, and mutations are observed in the subsequent F2 generation. Transposons are pieces of DNA that are capable

of moving autonomously in the genome. Mutations result from the insertion of a 'P' element into or near a gene or from the imprecise excision of a 'P' element, rearranging DNA in the vicinity of a gene. Mutagenesis by 'P' elements provides a method for cloning the gene into which a 'P' element has inserted been using the 'P' sequence as a molecular tag.

40,000 flies from the mutagenesis were screened, using the method of the deep pseudopupil (Franceschini 1971). From these a total of 14 alleles (independent mutations in the same gene) of *grb* were isolated. This high frequency indicates that *grb* is a hot spot for 'P' element insertion. 'P' elements are known to have sequence preferences for insertion. Stable lines were established by mating candidate mutant males to X^X 'P' cytotype females. In addition a number of homozygous (homologous chromosomes identical) lines were established.

The deep pseudopupil screen detects retinas in which the pattern of photoreceptor cells has not developed properly (Francescini, 1971). The screen makes use of the deep pseudopupil of the compound eye. Rhabdomeres, the light sensitive organelles of the photoreceptor cells, are oriented perpendicular to the retina. The deep pseudopupil is an image of the rhabdomeres, viewed by illuminating the *Drosophila* head capsule from behind as the flies are examined under an inverted microscope.

The wild-type (that of non-mutagenized populations)

pseudopupil is a sharp pattern of red dots in a trapezoidal array. The dots result from the contribution of light passing through the rhabdomeres of several adjacent ommatidia. The pseudopupils of *grb Drosophila* are variable between alleles and within each allele, ranging from a complete blur to a normal looking ommatidium with a slightly dim central cell.

ANATOMY

Transmission electron micrographs taken tangent to the eye surface of *grb* flies show the disorganization of the eye surface underlying the abnormal pseudopupil. In the most extreme cases the cells are completely disorganized. In the least extreme case cell R8 has not fully extended its rhabdomere into the center of the cluster, otherwise the clusters appear normal.

The structure of *grb* eyes and brains was examined using methylene blue and silver stained sections of paraffin embedded fly heads. The structure of the optic lobes appeared reasonably normal. At this gross level there was no apparent misrouting of axonal projections. Serial electron micrographs would be required to determine the state of the synaptic connections.

Figure 2:

Silver stained parrifin sections of *grb* brains showing fairly normal brain, axonal and retinal structure. Flies were processed in groups using the collar (Jager and Fischback, 1987), parrifin embedded, sectioned and silver stained (Blest, 1961).

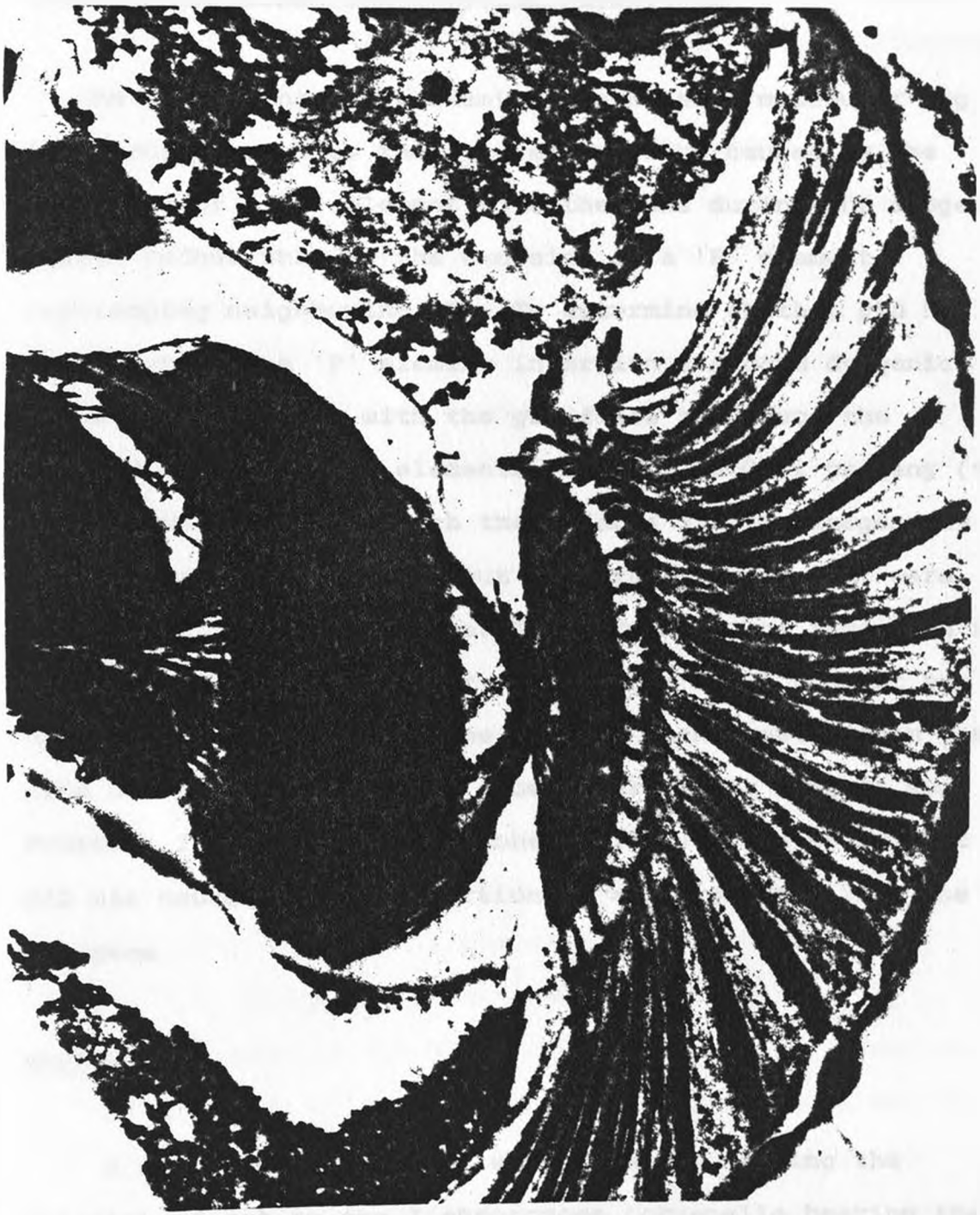


Figure 3:

Silver stained parrifin sections of wild type fly brains processed using the same method used for *grb* flies.

REMOBILIZATION AND ISOLATION OF REVERTANTS

The use of the 'P' element sequence as a molecular tag for cloning requires that the mutation be caused by the insertion of a 'P' element into the gene during the dysgenic cross, rather than by the excision of a 'P' element, rearranging neighboring DNA. To determine whether *grb* had been caused by a 'P' element insertion a second dysgenic cross was performed with the *grb* flies to induce the redistribution of 'P' elements. Out of 2,500 F2 progeny (the second generation in which the effects of the mutagenesis show up) of this cross, about 25 were obtained that were revertants to the wild-type. This frequency of reversion is like that reported in other cases where a 'P' element has been remobilized from a gene, and is much greater than the rate of reversion expected from other causes (Engels and Preston, 1979; Tsubota and Schedl, 1986). This shows that *grb* was caused by the insertion of a 'P' element into the *grb* gene.

MAPPING

A number of approaches were taken to mapping the position of *grb* on the X chromosome (organelle bearing the DNA, a long strand of DNA). Recombination (homologous chromosomes exchanging pieces during mitosis) mapping

studies were performed using the genetic markers (easily observeble mutations with known locations on the chromosome) on the X chromosome yellow (y), white (w), vermillion (v), and forked (f). Two alleles of *grb* were tested and the results of the two studies were in agreement. A total of 900 F2 male progeny were screened for recombinants.

Based on the recombination data alone *grb* is not on the X chromosome. The genetic distance between *grb* and each of the markers was the same, .46, .43, .44. Alternatively *grb* may be far proximal on the X chromosome in the region of heterochromatin.

In order to further attempt to map *grb* on the X chromosome a series of deficiency crosses were performed. The crosses made use of X chromososome transposition (pieces of chromosome rearranged) and deletion (piece of chromosome lost) strains. X chromosome deletions of overlapping segments of the X chromosome were used, together covering all of the X chromosome except about 20% of heterochromatin (tightly packed region of DNA near the base of the chromosome). Despite this thorough mapping of the X chromosome, none of the deletions uncovered *grb*. Based on this observation alone it seems likely that *grb* is not on the X chromosome. However breakpoints of deletions are determined using the cytological (observed in cells) banding patterns of chromosomes as landmarks. Banding is mostly absent in heterochromatin, so the breakpoints of the

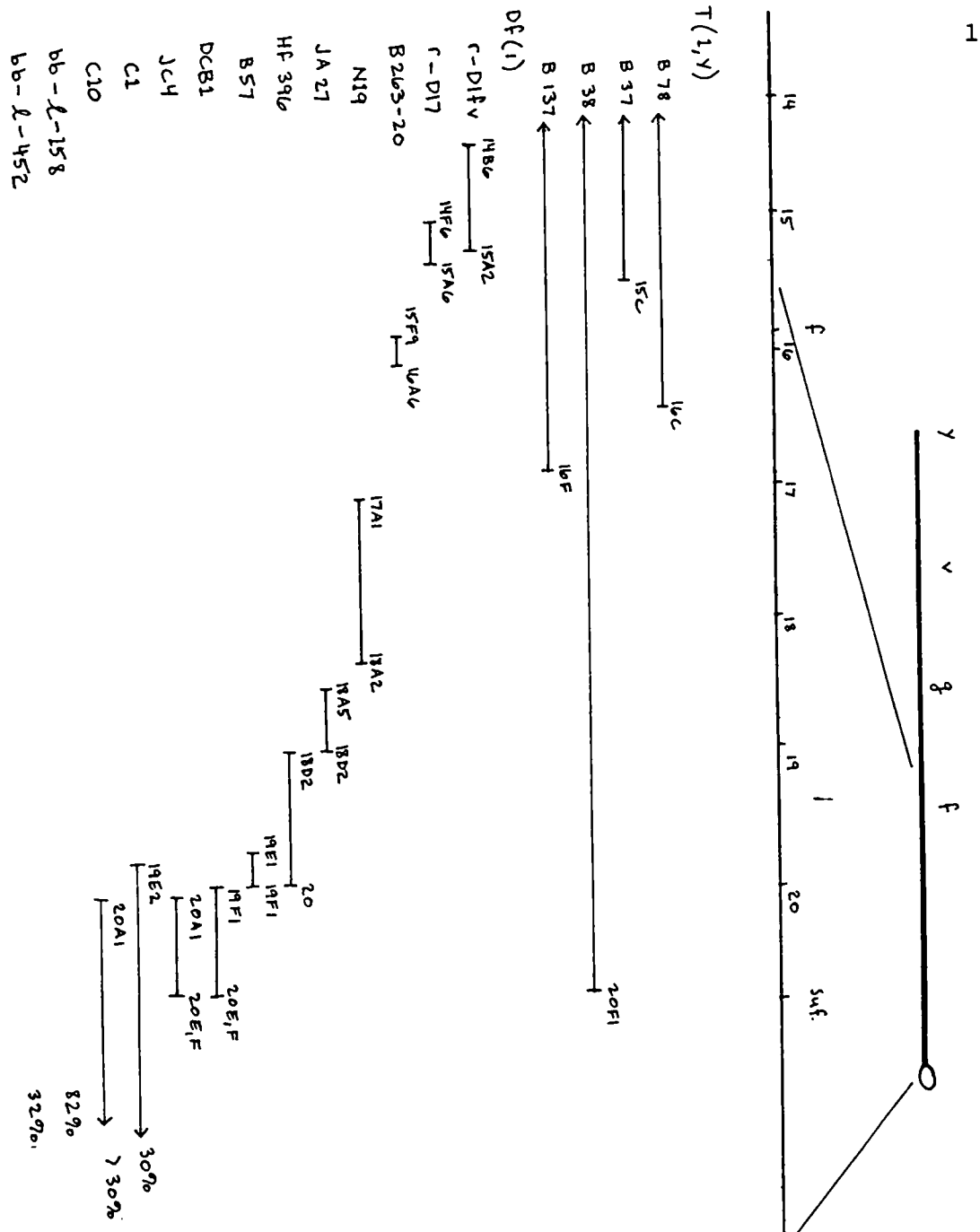


Figure 4:

Deficiency mapping. The regions of the X chromosome covered by each of the deletions used in the mapping are indicated on the diagram. Most of the X chromosome is covered excluding a portion of heterochromatin, on the right in the diagram.

deletions we used are not known with certainty. It may be

that less of heterochromatin was covered than we believe.

It is reported to be difficult to do molecular and genetic studies of genes in heterochromatin. Definite mapping in this region may require hybridization (probing for a molecule using another molecule with a structure complementary to the first) of 'P' element probes to salivary gland polytene (giant chromosome produced by many rounds of DNA replication without cell division) X chromosome squashes. Recombination mapping using genetic markers located more proximally may resolve the position of *grb* on the X chromosome. Similar crosses using labelled autosomes could be used to test for linkage between *grb* and one of the autosomes.

BEHAVIORAL STUDIES

Various kinds of mazes are commonly used to fractionate populations according to behaviors. Two methods were used to characterize the phototactic behavior of *grb* flies, a countercurrent apparatus for an initial crude measurement and a T-maze for a more refined measurement. Wavelengths and light intensities were better controlled in the T-maze experiments.

Seymour Benzer demonstrated the use of a countercurrent apparatus to fractionate a population of flies according to behavior (Benzer, 1975). He described the potential of this

approach for identifying various elements in a behavioral pathway by selecting for behavioral mutants. This method for fractionating populations according to behavior makes use of the relative attraction of flies between two behavioral alternatives. It is analogous to countercurrent distribution in chemistry, a method of separating molecules from a mixture by their partition between two solvent phases.

In this application, the flies are separated by being given successive opportunities to move toward a light stimulus. Separate tests were made for white light and UV light sources. Several alleles of *grb* were tested. For all studies of *grb*, Canton Special (cs) flies of the parental strain used in the mutagenesis were used for wild-type controls. Measurements with the countercurrent apparatus were more crude since wavelengths and light intensities were not controlled.

Unetherized flies in groups of about 100 were dark adapted for a few minutes. They were tapped to the bottom of the first chamber, which was slid into place opposite the first apposing tube. Illumination was from the distal end of the apposing tubes. After 15 seconds of phototaxis with intermittent tapping, the chambers were advanced relative to one another (Fig 1). The flies were given 2 minutes of rest in the dark before the next cycle was begun. After the completion of 6 cycles the number of flies each tube was

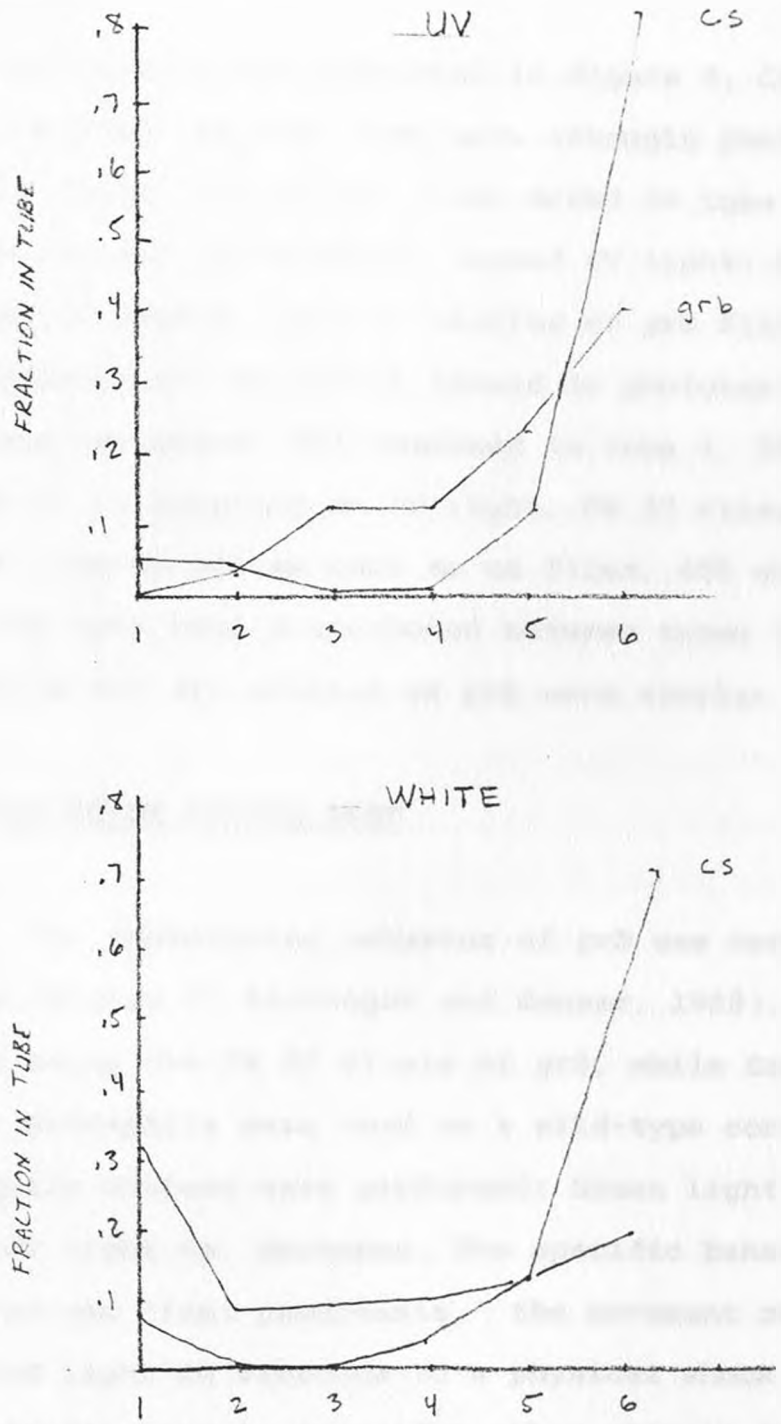


Figure 5:

Countercurrent measurements of phototaxis show *grb* to be less phototactic to green light relative to UV light as compared to wild-type.

counted.

The results are presented in Figure 6. CS flies behaved as one would expect. They were strongly phototactic toward white light: 70% of the flies ended in tube 6. They were also strongly phototactic toward UV light: 80% of the flies ended in tube 6. Initial studies of *grb* flies using the countercurrent apparatus showed no phototaxis. Later studies showed two peaks. 30% remained in tube 1, 20% were found in tube 6. In response to UV light, PW 37 flies phototaxed well, though not as well as cs flies, 45% wound up in tube 6, the rest were distributed between tubes 3, 4 and 5. The results for all alleles of *grb* were similar.

T-MAZE COLOR CHOICE TEST

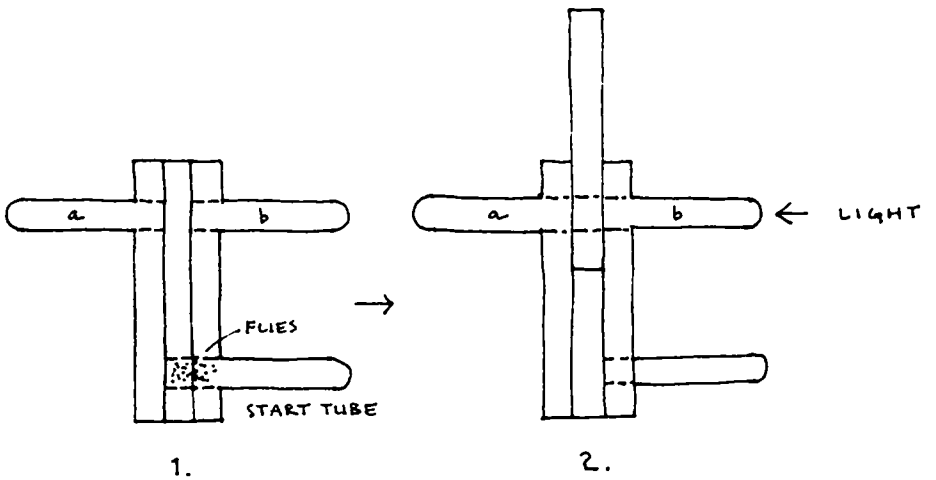
The phototactic behavior of *grb* was tested using the T-maze (Figure 7; Ballinger and Benzer, 1988). Most tests were done using the PW 37 allele of *grb*, while Canton Special (CS) *Drosophila* were used as a wild-type control. Two separate choices were performed: Green light vs. darkness and UV light vs. darkness. The specific behavior being tested was 'fast phototaxis,' the movement of *Drosophila* toward light in response to a physical shock. Fast phototaxis largely overrides other stimuli in the test environment.

Unetherized flies in groups of 30 - 50 were adapted to

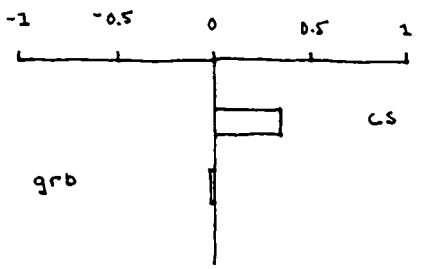
room light for at least 30 minutes. They were shaken from the start tube into a sliding chamber that was then moved into a central chamber between two 17 x 100 mm clear polystyrene test tubes. In a darkened room the apparatus was placed in a black paper lined box, one tube was illuminated from its far end through a portal. After 30 seconds of free running phototaxis with intermittent agitation by tapping the apparatus on a rubber pad, the choice tubes were isolated from one another and the flies in each tube counted. Green illumination was through a 550 nm narrow band interference filter with a 15W fiber optic source set at 1/4 intensity. UV illumination was from a 15W unfiltered source. Light intensities were not measured.

The T-maze results are reported as a phototactic index 1: 1(light/dark) = (na-nb)/(na+nb) where na is the number of flies in tube a and nb is the number in tube b. 1 = 1 indicates all the flies were attracted to the light source, 1 = 0 indicates the flies were divided evenly between the two sides.

For green light, 1 for *grb* = -0.02, and for CS is 0.35. CS flies moved well toward green light and PW37 flies did not at all. For UV light, 1 for *grb* is .92, and for CS is .94, essentially the same. By this measure, *grb* has a phototaxis defect that specifically affects the system that produces the green light response.



$$\lambda \text{ GREEN/DARK} = (na - nb)/(na + nb)$$



$$\lambda \text{ UV/DARK} = (na - nb)/(na + nb)$$

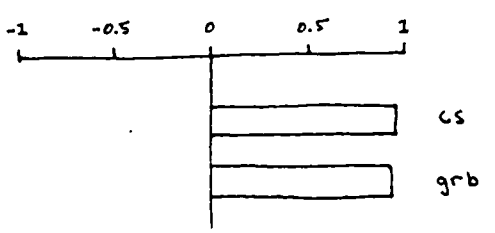


Figure 7:

T-maze phototaxis results show grb to be completely non-phototactic toward green light and normally phototactic toward UV light.

ELECTROPHYSIOLOGY

The electrical response of *grb* photoreceptor cells to light was measured using electroretinograms (ERG's) (Goldsmith, 1965). ERG's are a measure of the combined electrical response of the photoreceptor cells in a retina to light. They are performed by recording from electrodes implanted in the eye and head capsule of the flies. Within the limits of the method, the response of *grb* photoreceptor cells to green and to UV light was the same as that of wild type. However the on - off transient was significantly altered in mutant flies. The on - off transient is thought to represent a contribution from the optic lobes to the response, and may serve to indicate the state of neural connectivity between the photoreceptor cells and the optic lobes. The on - off transient in *grb* flies was greatly diminished compared to wild type.

DISCUSSION

All photoreceptor cell types are present in *grb* retinas, and all respond normally to light. However grb flies are behaviorally blind to green light but respond normally to UV light. This argues that the behavioral defect involves the processing of visual signals in the brain. *grb* has the properties expected of a mutant in which

the specific class of synaptic connections between photoreceptor cells R1 - R6 has failed to form properly. A mutation of this kind has not been described previously.

The *grb* locus (chromosomal site of the gene) and the mutant fly were examined in order to formulate a hypothesis for the role of *grb* in the development of the eye, and to provide the basis for further molecular and genetic studies of *grb*. *grb* was isolated as an X chromosome recessive mutation (both homologous chromosomes must be mutant to display the mutant character) from the progeny of a hybrid dysgenic cross (a type of cross that produces a high frequency of mutations). *grb* was isolated during a search for mutations affecting pattern formation in the retina using a screen that detects abnormal retina morphology. For further background information on this project please see the appendix.

Grb has the properties expected of a mutant in which a specific class of synaptic connections has not formed properly, those between photoreceptor cells R1 - R6 and neurons in the optic ganglia. *grb* is blind to green light but sees UV light, all of the photoreceptor cells are functionally intact. This argues that the defect is localized to this class of synaptic connections.

Two mutations affecting a specific synapse in *Drosophila* have previously been described. These were isolated using a specific search for such mutants in the

jump reflex of *Drosophila*. None have previously been described in the visual system.

BEHAVIORAL GENETICS

Behaviors are driven by groups of neurons interconnected in specific ways. This view has been investigated in greatest detail in the sea slug *Aplysia*, where various behaviors have been correlated with specific interconnections between small groups of neurons, and changes in behaviors with changes in neural connections. The current view is that in general the elements of behaviors are produced by groups of cells with specific patterns of connections between them.

Drosophila geneticists have been aware of behavioral variants in their fly populations for a long time. Seymour Benzer was the first to propose a systematic search for behavioral mutants as a means of identifying the components of behaviors and of their neural substrates (Benzer, 1975). He proposed the genetic dissection of behaviors and of the nervous system using single gene mutations to identify the elements of behaviors and of their neural substrates. He based this proposition on the success with which gene mutations had been used to delineate the complex network of interactions inside bacteria over the preceeding 20 years (Benzer, 1975).

Seymour Benzer pioneered the application of the mutational approach to the phototactic (phototaxis is the movement of an organism toward light) behavior of *Drosophila*, and demonstrated methods for isolating stably inheritable variants of phototactic behavior by making use of various maze arrangements. He reasoned that non-phototactic mutants would include a variety of types, those having defects affecting the various elements of the system. These might include mutations affecting the acquisition of cell fate (development of a cell type), visual transduction (cells transforming light energy to electrochemical response), neurotransmitter (molecules that signal between neurons) action and the formation of synaptic connections. Examples of many of these types have since been identified (Harris, 1976).

Drosophila is the principal organism for neurogenetic studies. *Drosophila* is ideally suited for genetic studies (Palka, 1983). The genetics are very well developed; *Drosophila* has been the main organism for genetic research for 80 years. There is a huge store of knowledge about the fly, a prerequisite for mutational studies. Recombinant DNA methods (in vitro manipulations of DNA) for *Drosophila* are at a high level of development (Roberts, 1986). For example transposable genetic elements provided a useful tool for manipulating the fly genome.

The mutational approach involves the production of

single gene mutations in flies that have otherwise identical genomes. Mutant flies are selected on the basis of predefined criteria. A model for how the process of interest works is used to define selection criteria that will recover interesting mutants. The mutations serve to identify the players (proteins) with key roles in the process being studied. By examining the way in which the process is altered in the mutant fly, the role of the protein and the developmental scheme in which it participates can be deduced. The hierarchical order of influence of these proteins, and their time and place of activity can be determined using genetic methods.

RECOMBINANT DNA STUDIES

Using recombinant DNA methods, genes and proteins identified by mutations can be studied at a molecular level. The example of *sev*, a mutation of Drosophila compound eye development, illustrates this kind of study well. The *sev* gene was cloned using transposon tagging, its nucleotide (DNA unit molecule) sequence determined, and shown to encode a hormone receptor-like transmembrane protein. *In situ* hybridization using oligonucleotide probes derived from the *sev* gene were used to determine the tissue distribution of *sev* RNA transcripts. Site directed mutagenesis was used to determine the molecular function of *sev*, a tyrosine kinase

domain (functional part of some molecules that receive extracellular signals) was shown to be critical to its function in R7 cell differentiation.

THE USE OF HYBRIDOMAS

Reverse genetics has also been used to identify eye development specific proteins. Monoclonal antibodies raised against Drosophila eye and brain were hybridized to developing eyes to identify development-specific proteins (Zipursky, 1988). Using reverse genetics the gene for one of these proteins, *chaoptin*, was recovered from a Drosophila genomic library, this done using an oligonucleotide probe derived from a portion of the N terminus of the protein. This was followed by the usual molecular studies.

CONNECTIVITY STUDIES IN THE COMPOUND EYE

Because of its versatility for molecular and genetic studies, the compound eye has been used to study genes and proteins not directly related to compound eye development. Human oncogenes (genes which when mutated in certain ways produce cancers) have been studied in the eyes of transformed flies (in which foreign DNA has been added to the genome), as has the *Drosophila* neurogenic gene *notch*. *Notch* mutations lead to hypertrophy of neural tissue.

Studies of neural connectivity can benefit from the level of analysis possible in the compound eye and by the accumulation of knowledge generated by research aimed at aspects of compound eye development like pattern formation and neuronal differentiation. In the compound eye synaptogenesis can be studied as an aspect of neuronal differentiation. Synaptogenesis is seen as a family of steps beginning with nerve cell differentiation and ending with the forming of a functioning synapse. Each step is composed of subroutines, each carried out by molecules acting within the cell. As such synaptogenesis is a problem of neuronal differentiation and many processes will be common to both.

To some degree studies of pattern formation are already providing clues about synaptogenesis and axonal pathfinding. *Chaoptin* is an example of this, as may be *grb*. Chaoptin is involved in adhesion between the surfaces of adjacent neurons. Chaoptin is also present on the surfaces of growing photoreceptor cell axons, suggesting a possible role in axonal guidance *via* adhesion.

In some respects the behaviors mediated by the compound eye are less suited for studies of neural connectivity than is the jump reflex. The neurons driving these behaviors are much smaller; it is not possible to perform dye injections or intracellular recording.

AXONAL GROWTH

The compound eye is a striking example of axonal guidance and synaptic specificity. Because of the curvature of the eye and the orientation of the rhabdomeres growing axons must follow precise, interweaving pathways. Their growth occurs in such a way that the visual field is topographically projected onto the optic ganglia. The axon termini form a precise pattern in the optic lobes, with each axon type terminating in a characteristic position.

Axons originate from the proximal tips of the photoreceptor cells during the third larval instar and pupariation. R8, the first cell to differentiate, is the first to originate an axon. The axons of the other R cells follow sequentially in a tight bundle around that of R8.

Contact from the photoreceptor cell axons is required for the formation of the structure of the optic lobes (Kandel and Meyerowitz, 1978). In the absence of such contact, optic lobe neurons fail to differentiate and their growth is chaotic. Growing photoreceptor cell axons arrive at the optic lobes in a sequence corresponding to that of photoreceptor cell differentiation, anterior to posterior and R8 followed by the others in order. The sequential arrival is probably instrumental to the formation of the structure of the optic lobes.

Axons are thought to be guided in their growth by

molecular cues. These may be molecules distributed on cell surfaces, on the extracellular matrix or as diffusable substances. The eye is a somewhat different case than the Drosophila jump reflex. Wyman and Thomas reasoned that selective pressure on evasion behavior would have tailored the jump response such that there would be many genes specific to the reflex, and a high probability of finding mutants specific to the single synapse in the pathway. To code for a molecule uniquely specifying each connection in the fly would exceed the size of the genome. The eye consists of repeated functional modules and sheets of neurons. In the compound eye positional information may be given by molecules acting in a modular fashion and by diffusion across sheets of cells.

PROPOSED STUDIES

Based on the studies described above *grb*, involves a phototaxis defect localized to the synaptic connections between cells R1-R6 and their synaptic targets in the optic lobes. Previous studies have identified two similar gene mutations affecting the jump reflex of Drosophila, while none have previously been described in the eye. The visual system has unique advantages for the study of neural connectivity. Further studies should be performed to determine the nature of the *grb* mutation and the role of the

grb protein in visual system development and function. A systematic search for more mutants of this class in the visual system should be performed.

NULL ALLELE OF *GRB*

It is important to isolate a null allele of *grb* (in which the gene is completely inactivated by a mutation). The considerable variation of the phenotype within each allele of *grb* suggests that in all of them the gene is only partially inactivated. The null allele may have a completely different phenotype, which would give a different view of the wild-type gene function. 13 alleles of *grb* were recovered. If none among these were nulls this would suggest that a null mutation was lethal. Several genetic tests for null alleles can be performed. For a null allele, the homozygous mutant should have the same phenotype as a fly heterozygous (homologous chromosomes are unalike) for the mutation and a deletion covering the same region of the chromosome. A second method for determining the null phenotype involves producing heterozygotes with two deletions that overlap at the site of *grb*. Both of these involve straightforward genetic crosses.

MOSAIC STUDIES

It would be informative to determine in what tissues the *grb* wild-type protein is required for normal function. This would provide important clues regarding the role of *grb* in axonal pathfinding and synaptogenesis. A genetic means for accomplishing this involves the production of flies that are mosaic for the mutant and wild-type genes (Hotta and Benzer, 1978). In Drosophila genetic mosaics can be produced by using X-irradiation to induce somatic recombination in cells undergoing mitosis. By irradiating a +/- cell, daughter cells that are +/+ and -/- are produced. Cells in which recombination occurs early in their lineage result in large clones (population of genotypically identical cells) of marked cells. In such flies the optic lobes could be mutant and the retina wild type or *visa versa* (Meyerowitz and Kankel, 1977). Where the clonal boundaries pass through the eye, ommatidia with both wild type and mutant cells are found (Ready, 1976). These combinations could be used to differentiate a requirement for the *grb* product in the optic lobes, in cells R1 - R6 or in cell R8, for example. The developmental fates of cells marked in this fashion can be followed. Whether the mutant phenotype were produced when R8 cells, R1-R6 cells, optic lobe neurons or some other cells were mutant would suggest totally different roles for *grb*.

SYSTEMATIC SEARCH FOR *GRB*-LIKE MUTANTS

A systematic search should be undertaken for the entire class of mutants altering synaptic connectivity between R1-R6 and the optic lobes. Wyman and Thomas demonstrated how a series of screens could be used to accomplish this in the jump reflex. The search would be conducted in an analagous manner for *grb*.

From a mutagenized population of flies, those lacking green light phototaxis would be selected using the countercurrent apparatus. From among the progeny of these, those retaining UV light phototaxis would be selected, again using the countercurrent apparatus. From among these, those with an intact pseudopupil, indicating the presence of all photoreceptor cell types, would be selected. Finally, from among these, those with normal R1 - R6 cell function would be selected by measuring the response to green light using electroretinograms.

A similar search by Wyman and Thomas applied to one synapse in the jump reflex of *Drosophila* yielded two mutations acting in different ways (Wyman and Thomas, 1983). In the mutant *Passover* the Giant Fiber is misrouted, and the synapse fails to form. *Bendless* primarily affects the jump motor neuron (neuron that synapses with muscle), the synaptic partner of the Giant Fiber. Several classes of connectivity mutants in the green light response could be

found, primarily affecting the photoreceptor axons, the pathways along which they grow or the neurons in the optic lobes.

MOLECULAR CLONING OF *GRB*

It is important to determine the molecular role of *grb* in axonal pathfinding and synaptogenesis. This can be accomplished *via* the molecular cloning of the gene. Since *grb* resulted from a 'P' element insertion into the gene, a 'P' element homologous probe can be used to identify clones bearing the *grb* gene from a *grb* genomic library.

Growing axons are thought to be guided by molecular cues (Letourneau, 1975; Goodman, 1984). The nature of these molecules is not known. The tip of a growing axon is an active structure (a growth cone) that appears to sample its environment and selectively adheres to certain surfaces. Selective adhesion of this sort may be instrumental in axonal guidance. A group of cell adhesion glycoproteins (proteins modified with sugar residues) have been isolated that may play this role. These include nerve growth factor (NGF), nerve cell adhesion molecules (N-CAMS) and fibronectin. The molecular heterogeneity of these would allow them to specify many pathways. Glycoproteins have sialic acid sugar residues that can be tailored in many ways.

Chaoptin was identified using a screen of anti sera (serum containing antibodies) to Drosophila eye and brain. *Chaoptin* is present at the furrow, the site of active differentiation in the imaginal disc, and on the surfaces of growing axons. *Chaoptin* encodes a cell surface glycoprotein. It may be that *grb* does also.

The cloning and molecular characterization of *grb* would follow procedures that are well established for *Drosophila*. Recombinant DNA methods can be used to add a level of analysis beyond what is possible with genetics. The study of the eye mutant *sev* provides the best example of this approach. *Sev* is a mutation that alters the *Drosophila* retina in a precise fashion. In *sev* flies, one photoreceptor type is absent. *Sev* was isolated using a behavioral screen; *sev* flies fail to move toward UV light.

Cloning refers to the isolation of a piece of DNA on a vector (carrier, a route of transmission), frequently a virus (noncellular organism made of nucleic acid and protein), grown up as an autonomous piece of DNA in a host cell type, usually bacteria. An outline of the method of cloning *sev* follows.

An allele of *sev* that was produced by a transposon insertion, as in the case of *grb*, was used to clone the *sev* gene. *Sev* DNA was isolated and the *sev* genome was cut into fragments using restriction enzymes (enzymes that cut nucleic acid polymers at specific sites). Approximately

equal-sized fragments were packaged into virus protein coats by ligating specific viral signal sequences to the DNA fragments. The virus DNA has specific base pair sequences that promote packing of the virus DNA into the protein capsid. The resulting population of virus constructs contained heterogenous pieces of the *sev* genome, together comprising the entire genome. These were used to infect a population of *Escherichia coli*. In this fashion the entire *sev* genome was cloned in fragments into *E. coli*

The next step was to identify the clone containing DNA of the *sev* gene. This was done in the following fashion. A dilute suspension of the virus was plated onto a lawn of *E. coli* on flat agar plates. A radioactive cDNA (a strand of DNA with a sequence complementary to the parent strand of DNA from which it was synthetically copied) probe with a sequence complementary to that of the transposon was applied to the surface of the plates. The probe hybridized specifically to DNA on the plate bearing the transposon sequence, and was detected by autoradiography (used to develop a film covered with photographic emulsion), indicating the location of E.coli populations bearing *sev* genomic DNA with transposon sequences in them. These clones constituted candidates for *E. coli* carrying all or part of the *sev* gene.

The next step was to determine which of the clones bearing transposon DNA had DNA from the *sev* locus. The

candidate clones were separately used to produce labelled cDNA's. cDNA from each clone was hybridized to polytene chromosome squashes (*Drosophila* have giant chromosomes in their salivary glands that can be viewed under the light microscope) from wild type *Drosophila*. Those cDNA's with a sequence flanking the transposon sequence that originated from the *sev* locus hybridized to that site on the squashes and were detected by autoradiography. In this fashion one or several clones containing some or all of the *sev* gene were identified.

The cloning of *sev* provided the entry for a number of studies that have been conducted subsequently. These include the localization of the protein, determination of the gene and protein sequence, comparison of *sev* with known proteins to formulate a hypothesis about the molecular function of *sev* and site directed mutagenesis of *sev* to test the predicted function. The predicted sequence of the *sev* protein was compared to that of proteins with known functions and found to be similar to that of transmembrane proteins with hormone receptor functions. The distribution of both the *sev* protein and the *sev* RNA transcripts was determined by hybridization. Studies of this kind would be the aim of further work with *grb*.

APPENDIX

STATEMENT OF PURPOSE

As an independent study project this project has been intended to serve clear educational objectives. My purpose has been to examine a current trend in biology, the application of molecular biology methods and techniques to many branches of biology, in particular in this case to neurobiology. I have inquired what the current questions in molecular neurobiology are, what methods are being used to address them and how the methods are creatively applied to answering the questions. The approach I have taken is a practical one, by undertaking a laboratory research project I have obtained some answers to these questions. The specific research project has been the characterization of a *Drosophila Melanogaster* (fruit fly) visual system mutant.

THE CONTEXT FOR THE PROJECT

In order to make this project more meaningful to a lay reader I am providing this overview of the development of the field of molecular neurobiology, since the quesions and methods described make better sense in this context.

The application of molecular biology methods to neurobiology dates from the late 1960's, when a number of

individuals migrated from the former field into the latter, seeking to apply their methods to the basic unsolved questions of neurobiology. The aim of molecular biology has been the explanation of biological processes at the level of molecules and their interactions. The aim of neurobiology has been an understanding of the function of neurons, and of how they produce cognition, memory perception and so forth.

Molecular biology had its origins in the study of a number of problems. Thomas Hunt Morgan, the geneticist, used *Drosophila* to investigate the molecular basis of inheritance in higher organisms. Max Delbruck, the emigre' physicist, adopted bacterial viruses as a system for studying the molecular basis of the replication of genetic material. A British physicist, Lawrence Bragg, applied X-ray crystalography to the study of keratin and nhemoglobin in order to understand the properties of biological polymers. The regulation of cell metabolism was studied using bacteria and fungi by Beadle and Tatum (two American biochemists) Jacob and Monod (two French virologists) and others. By the late 1970's a framework of answers to these basic questions had been established. Chromosomes had been shown to bear the genetic material. Genes had been shown to act by coding for polypeptides. The structure of DNA had been established, and a basic description of how it works had been outlined. The operon hypothesis had provided a model for how genes are organized and coordinately regulated as a way of integrating

cell activities.

The principles molecular biologists had used to study these problems were brought with them in their migration to neurobiology. These principles can be outlined as follows. They used a model building approach, proceeding by proposing and testing hypotheses, in contrast to a mainly descriptive approach. While biologists were interested in complex phenomena, they selected simple systems for their study. Bacterial viruses, fruit flies and fungi exemplify this. This strategy is based on the assumption that biological processes are universal, so that what is learned in one system will be applicable to others. Mutational genetics, a mainstay of molecular biology, was introduced as a novelty to neurobiology. In addition, more recently, the powerful techniques of recombinant DNA and hybridomas have become available.

The migration has transformed neurobiology in a number of ways. The language of neurobiology has until recently been centered largely on detailed descriptive studies of electrophysiology and neuroanatomy. It is now becoming more firmly grounded in the more universal languages of biochemistry and molecular biology. This is indicative of a general erosion of the barriers between cell biology and neurobiology. Since the techniques can be applied to any preparation, there is the opportunity for biologists to move between fields. Individuals coming from outside neurobiology

are finding some of the traditional problems of neurobiology approachable, and neurobiologists are finding that some of their questions can best be addressed in other systems.

The single gene mutation approach as a means for functionally dissecting the nervous system has since been applied to a wide range of behaviors and organisms. Behaviors to which it has been applied include olfaction (smell), learning (behavior altered by experience) and memory (neural record of experience) and courtship (Hall, 1978; Harris, 1976; Rodrigues and Siddiqi, 1978; Konopka and Benzer, 1971). New genetic systems have been developed specifically for this kind of study. Isogenic strains (in which the genome is uniform and invariant, making the production of single gene mutants possible) of fish and mice have been developed mainly to study neural connectivity. The nematode (worm) *cenerhobditis elegans* has the advantage of an invariant cell population in which every cell has been identified.

Some of the questions that should be accessible to molecular neurobiology are the following. There is an incredible diversity of macromolecules in the brain as compared to other tissues, how is this produced? There is a huge diversity of cell types, as differentiated by morphology, position, connectivity, proteins expressed (for example transmitters and channels). What constitutes a cell type in the brain, what is the order to this diversity? What

is the association between gene expression and behaviors, for example learning and cognition? What is the molecular basis for the formation of the appropriate arrangement of cells of diverse types in the nervous system? How is axonal pathfinding and synaptogenesis (the formation of connections between neurons) governed at a molecular level?

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